Elizabeth de Abreu Lopes

Licenciatura em Química

Spirooxadiazoline oxindoles: synthesis and evaluation of anticancer and antimalarial activities

Dissertação para obtenção do Grau de Mestre em Química Bioorgânica

Orientador: Maria M. M. Santos, Ph.D, FFULisboa

Co-orientador: Lídia M. Gonçalves, Ph.D, FFULisboa

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro, 2017

Elizabeth de Abreu Lopes

Licenciatura em Química

Spirooxadiazoline oxindoles: synthesis and evaluation of anticancer and antimalarial activities

Dissertação para obtenção do Grau de Mestre em
Química Bioorgânica

Orientador: Maria M. M. Santos, Ph.D, FFULisboa
Co-orientador: Lídia M. Gonçalves, Ph.D, FFULisboa

Presidente: Prof. Doutora Paula Cristina Sério Branco, FCT-UNL
Arguente(s): Doutor Vasco Daniel Bigas Bonifácio, IST-CQFM
Vogal(ais): Prof. Doutora Maria M. M. Santos, FFULisboa

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro, 2017

Spirooxadiazoline oxindoles: synthesis and evaluation of anticancer and antimalarial activities

Dedico esta tese ao meu pai, uma das pessoas mais importantes da minha vida e de quem as saudades são cada vez maiores. Se não fosse por ti, nunca teria chegado até aqui. Talvez não tenhas vivido e não tenhas feito tudo o que querias fazer, para eu pudesse escrever esta tese hoje e por isso agradeço-te, por todo o sacrifício. Gostava que estivesses aqui para ver até onde cheguei e até onde vou chegar. Obrigada por tudo. Amo-te

.

Poster communications

Lopes E., Uyar D., Gonçalves L., Santos M. M. M. "Spirooxadiazoline oxindoles as anticancer agents" 9th IMed.ULisboa Postgraduated Students Meeting & 2nd i3du Meeting. Lisboa, Portugal. 13-14/07/2017

Lopes E., Uyar D., Gonçalves L., Santos M. M. M. "Synthesis of novel spirooxadiazoline oxindoles and evaluation as anticancer agents" XXV Encontro Nacional da Sociedade Portuguesa de Química. Lisboa, Portugal. 16-19/07/2017

Lopes E., Uyar D., Gonçalves L., Santos M. M. M. "Spirooxadiazoline oxindoles: potential anticancer agentes" 1º Encontro do Colégio da Química. Lisboa, Portugal. 20-21/07/2017

Lopes E., Uyar D., Gonçalves L., Santos M. M. M. "Spirooxadiazoline oxindoles – a novel family of anticancer agents" 2nd Training School of Cost Action CM1407. Lisboa, Portugal. 18-20/09/2017.

Lopes E., Uyar D., Gonçalves L., Santos M. M. M. "Spirooxadiazoline oxindoles – a novel family of anticancer agents" 4th Meeting of Cost Action CM1407. Lisboa, Portugal. 21-22/09/2017

Table of contents

Table of contents

Figure index

Figure index

Table index

Scheme index

Agradecimentos

Em primeiro lugar, quero agradecer à minha orientadora, Dra. Maria M. M. Santos, por me ter aceite no seu grupo e por todo o apoio, críticas e exigência que me tornaram melhor química do que alguma vez fui. Agradeço à minha co-orientadora, Dra. Lídia Gonçalves, por toda a ajuda nos ensaios biológicos e ao grupo de investigação do Dr. Philip Rosenthal por ter realizado os ensaios biológicos de *P. falciparum*. Os meus agradecimentos estendem-se ainda à Dra. Noélia Duarte e Dra. Paula Nobre pelos ensaios de LC-MS.

Agradeço também aos meus colegas da "MS Team", Dário Silva, Valentina Barcherini, Margarida Espadinha e Alessandro Liteanu, por todo o apoio, bom ambiente e ajuda durante este ano. Um agradecimento especial para a Margarida por todas as discussões e ajuda no laboratório quando a inspiração desaparecia.

Neste seguimento, agradeço também ao Jorge Grilo pela preciosa ajuda em todas as vezes que precisava de iluminação racional.

Agradeço ainda à Ana Rita Pratas e à Lara Fidalgo por todos os jantares e copos durante este ano e todas as corridas matinais para começar o dia, mesmo quando a preguiça era demasiada.

Obrigada a todos os colegas de laboratório por tornarem este ano muito mais agradável, com todas as brincadeiras, companheirismo e cumplicidade que não se encontram em mais lado nenhum, inclusive ao Colega (ele sabe quem é) por todas as imitações que, no fundo, faziam-me rir.

Fora do círculo da faculdade, agradeço à Carolina Queiroz e à Mariana Reis, as minhas colegas de casa, pela amizade e companheirismo que pode ser muito difícil encontrar quando se mora fora de casa.

Obrigada também ao meu grupo de amigos em Viana (Ricky, Freixo, Baptista, Motas, Vieira e Cuco) por todos os bons momentos e por toda a cumplicidade demonstrada desde o 12º ano. E ainda, à Jéssica Fernandes pelo simples fato de ser minha amiga e estar ao meu lado nos momentos mais difíceis.

Por último, mas não menos importante, queria agradecer à minha família, em especial à minha mãe por todo o esforço financeiro que tem feito para sustentar-nos sozinha, assim como por todo o apoio nestes últimos dois anos que têm sido tão difíceis. Ao meu irmão por ser simplesmente meu irmão e aos meus avós paternos por toda a ajuda que vêm dando desde o meu primeiro ano de faculdade.

A todos muito obrigada.

Abbreviations

3D7	*Plasmodium falciparum* chloroquine-sensitive strain
µM	Micromolar
Å	Ångström
ACN	Acetonitrile
AML	Acute myeloid leukaemia
Asn	Aspartame
Asp	Aspartate
ATP	Adenosine triphosphate
br	Broadened
Cmpd	Compound
cyB	Cytochrome B
Cys	Cysteine
d	Doublet
dd	Doublet of doublets
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
dt	Doublet of triplets
EC_{50}	Concentration of 50% of maximal effectiveness
Equiv	Equivalents
ERBB2	Erb-b2 receptor tyrosine kinase 2
ESI	Electrospray ionization
Et	Ethyl
EtOAc	Ethyl acetate
FDA	Food and Drug Administration
Fig.	Figure
G1 phase	Growth 1/Gap 1 phase
GI_{50}	Concentration for 50% of maximal inhibition of cell proliferation
Gln	Glutamine
HCT116 *p53*$^{(+/+)}$	Human colorectal cancer cell line with wild-type p53
His	Histidine
HIV	Human immunodeficiency virus

Abbreviations

Hz	Hertz
IC_{50}	Concentration for 50% of maximal inhibition
Ile	Isoleucine
IR	Infra-red
J	Coupling constant
K1	*Plasmodium falciparum* multidrug-resistant strain
kDa	kiloDalton
LC	Liquid chromatography
Leu	Leucine
m	Multiplet
MCF-7	Human breast adenocarcinoma cell line
MDA-MB-231	Human breast adenocarcinoma cell line
MDM2	Minute Double Murine 2
MDMX	Minute Double Murine X
Met	Methionine
MHz	Megahertz
mM	Millimolar
MP	Melting point
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
***Mut* p53**	Mutated p53
MW	Microwave
NCS	N-chlorosuccinimide
NMR	Nuclear Magnetic Resonance
NP	Natural products
ºC	Celsius degrees
P.	*Plasmodium*
Ph	Phenyl
Phe	Phenylalanine
PMM	Pentamethylmelamine
Ppm	Parts per million
Pro	Proline
p-TLC	Preparative thin layer chromatography
q	Quartet
RBC	Red blood cells

RNA	Ribonucleic acid
s	Singlet
SAR	Structure-activity relationship
SD	Standard deviation
Ser	Serine
SW620	Human colorectal cancer cell line with mutated p53
t	triplet
TEA	Triethylamine
Thr	Threonine
TLC	Thin-layer chromatography
Trp	Tryptophan
Tyr	Tyrosine
UK	United Kingdom
USA	United States of America
UV-Vis	Ultraviolet-visible
Val	Valine
W2	*Plasmodium falciparum* chloroquine-resistant strain
WHO	World Health Organization
***Wt* p53**	Wild type p53

Abstract

Cancer is one of the modern world's most common and deadly non-infectious disease. According to WHO Cancer Report of 2015, it is one of leading causes of morbidity and mortality worldwide with 8.8 million deaths in 2015 and it is expected to rise about 70% over the next 20 years. The non-selectivity and acute toxicity of many anticancer agents has prompted the search for new alternatives with improved tumour selectivity, efficiency and safety.

Spirooxindole alkaloids are a family of natural products that have a spiro ring fusion at position 3 of the oxindole core. Several natural products that possess this heterocyclic core, such as alstonisine, horsfiline, strychnofoline and spirotryprostatin A and are described as having interesting bioactivities. These scaffolds have been described with different biological activities, including *in vitro* anticancer activities in several cancer cell lines.

In this thesis, we report the development of a novel library of spirooxadiazoline oxindoles derivatives with an [1,3,4]-oxadiazole ring and evaluation of its anticancer and antimalarial activities. With this goal, twenty spirooxadiazoline oxindole compounds were synthesized by reaction of isatin derivatives and nitrile imines (formed *in situ* from the corresponding hydrazonyl chlorides), in moderate to high yields (42-90%).

The anti-proliferation activity of this library was tested in two breast cancer cell lines, MCF-7 and MDA-MB-231, at a concentration of 50 μM. In MCF-7, the p53 retains wild type conformation and in MDA-MB-231, the p53 protein has the mutation R280K. Five compounds showed to inhibit around 40-50% the cancer cells growth, at 50 μM concentration. Two of them showed to act in both tested cell lines, other two compounds showed to be more selective for MDA-MB-231 cell line and one showed to be selective for MCF-7 cell line. These five compounds show the importance of some substituents such as the *meta*-substituted phenyl ring in position 3', the substitution in position 5 and the alkyl group in position 5'.

Comparing the anti-proliferation activity of this library with its regioisomer, the [1,2,4]-oxadiazole scaffold shows higher inhibition of cancer cells growth than the [1,3,4]-oxadiazole scaffold, for both cancer lines. This can be explained by the different position of the substituents in space that fill the binding site.

It has been reported that some compounds, for example artemisinin, have both anticancer and antimalarial activity. For this reason, this family was also tested in *Plasmodium falciparum* strains, W2 and 3D7. Six compounds showed to be very potent for a chloroquine-resistant strain (W2), with IC_{50} values between 1.4 and 9.0 μM. In addition, two compounds showed to be potent for both chloroquine resistant and sensitive strains, W2 and 3D7, respectively.

Overall, in this work it is reported for the first time the synthesis of a new family of spirooxadiazoline oxindoles. It is also the first time reported 1,3-dipolar cycloadditions by

Abstract

reacting the hydrazonyl chlorides with isatin derivatives, reducing the number of reaction steps. The anti-proliferative activity is also reported for the first time in two breast cancer cell lines and, more importantly, the discovery that these small molecules represent useful compounds for the development of novel antimalarials.

Keywords: spirooxadiazoline oxindoles; 1,3-dipolar cycloaddition; breast cancer; p53; malaria

Resumo

O cancro é a doença não infeciosa mais comum e mortal (a nível mundial). De acordo com o relatório de 2015 da Organização Mundial de Saúde, é uma das principais causas de mortalidade em todo o mundo, com cerca de 8.8 milhões de mortes em 2015, sendo expectável que aumente 70% nos próximos 20 anos. A falta de seletividade e toxicidade de vários agentes anticancerígenos tem levado à pesquisa de novos agentes que tenham seletividade para células cancerígenas sem comprometer a sua eficácia e toxicidade.

Os alcalóides spirooxindóis são uma família de produtos naturais que contêm um carbono spiro na posição 3 do oxindole. Vários produtos naturais têm esta estrutura heterocíclica, tais como a alstonisina, a horsfilina, a strichnofolina e a spirotriprostatina A. Os spirooxindóis têm sido descritos como tendo diferentes atividades biológicas, incluindo atividade anticancerígena sobre diversas linhas celulares.

Durante o trabalho experimental desta tese, sintetizou-se uma biblioteca de vinte novos derivados de spirooxadiazolina oxindóis. Estes compostos foram posteriormente submetidos a ensaios de anti-proliferação em linhas celulares de cancro da mama, MCF-7 e MDA-MB-231, e a ensaios contra *P. falciparum*. Esta família de spirooxadiazolina oxindóis, com um núcleo de [1,3,4]-oxadiazolina, foi sintetizada a partir de derivados de isatina e nitrilo iminas (formadas *in situ* a partir dos cloretos de hidrazonilo correspondentes), com rendimentos moderados a elevados (42-90%).

A atividade anti-proliferativa desta biblioteca foi avaliada em duas linhas celulares de cancro da mama, MCF-7 e MDA-MB-231, numa concentração de 50 µM. Na linha MCF-7, a p53 mantém a conformação nativa e na linha MDA-MB-231, a p53 está mutada. Cinco compostos mostraram 40-50% de inibição do crescimento de células cancerígenas, a uma concentração de 50 µM. Dois compostos mostraram atuar nas duas linhas celulares testadas, outros dois compostos mostraram seletividade para a linha celular MDA-MB-231 e um composto mostrou seletividade para a linha celular MCF-7. Estes compostos têm substituintes semelhantes, tais como, o anel aromático da posição 3' substituído na posição *meta*, a substituição do anel indólico na posição 5 e o grupo alquilo na posição 5', o que leva a crer que são importantes para a interação com o alvo biológico.

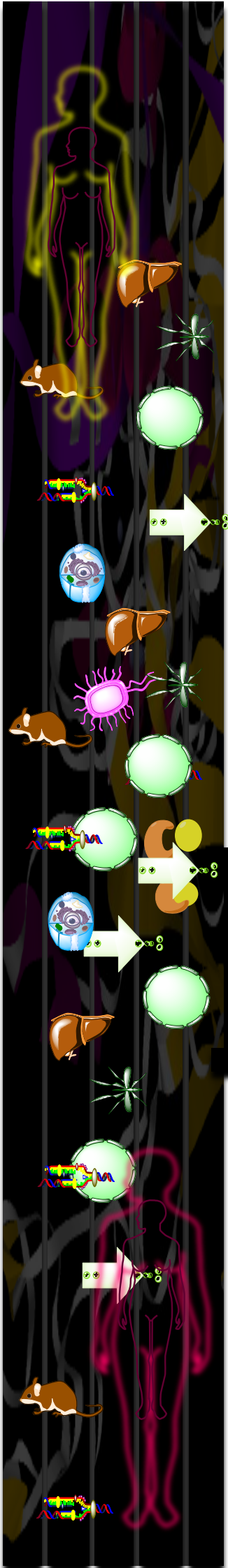
Comparando as atividades anti-proliferativas desta família com spirooxadiazolina oxindole com um núcleo de [1,2,4]-oxadiazolina (regioisómero), este último tem um maior taxa de inibição de crescimento de células cancerígenas. A maior diferença entre os regioisómeros é a posição dos substituintes no espaço que preenchem o sítio ativo.

Têm sido descritos alguns compostos, como por exemplo a artemisina, que têm atividade anticancerígena e antimalárica. Por esta razão, as spirooxadiazolina oxindóis foram também

testadas em estirpes de *Plasmodium falciparum*, W2 e 3D7. Seis compostos mostraram ser muito potentes para a estirpe resistente à cloroquina (W2), com valores de IC_{50} entre 1.4 e 9.0 μM. Adicionalmente, dois compostos mostraram ser potentes tanto para a estirpe resistente como para a estirpe sensível à cloroquina, W2 e 3D7, respetivamente, alargando o espetro de atividade da família.

Em resumo, nesta tese é descrita pela primeira vez a síntese de uma nova família de spirooxadiazolina oxindóis, a sua atividade anti-proliferativa em duas linhas celulares de cancro da mama e, mais importante, a descoberta que estes compostos têm potencial para o desenvolvimento de novos agentes antimaláricos.

Palavras-chave: spirooxadiazolina oxindóis; cicloadição 1,3-dipolar; cancro da mama; p53; malária



Chapter 1

NATURAL PRODUCTS: INDOLES AND SPIROOXINDOLES

Chapter 1. Natural products: indoles and spirooxindoles

Nature has been a valuable source of compounds to develop drugs.[1] The extracts of the plants represent a large family of chemical entities with several biological activities such as anticancer, antimalarial, antiviral and antipyretic **1**-**5**. They are originated mostly from bacteria, fungi, plants and marine organisms.[2]

According to WHO, in 1985, approximately 65% of the world population had plant-derived traditional medicine for their primary health care. Since the discovery of penicillin, in 1928, more than 23000 natural products have been characterized. Most of them are from bacterial source, mainly from the family *Actinomycetaceae*. After the discovery of streptomycin by the Selman Waksman group at Rutgers University, a lot of efforts in pharmaceutical companies and academic laboratories have been made for natural products (NPs) discovery. [3, 4]

NPs have exceptional characteristics such as chemical diversity and significant number of stereospecific carbon centres. This last feature attracts synthetic chemists interested in developing routes for total synthesis.

Until 2013, 1453 new chemical entities have been approved by FDA, which 40% are natural products or their derivatives. This number has risen 50% in the last 30 years, from which 74% are for anticancer applications. However, the anti-infective therapy is the area of highest use of NPs. For example, artemisinin (**1**) is a NP, widely used as an antimalarial drug and it is produced by the yeast *Saccharomyces cerevisiae*. Quinine (**5**) is another antimalarial drug, first isolated from the bark of Cinchona species and reported in 1820. The bark was used by indigenous groups from Amazon for the treatment of fevers (**Fig. 1.1**).[3, 5]

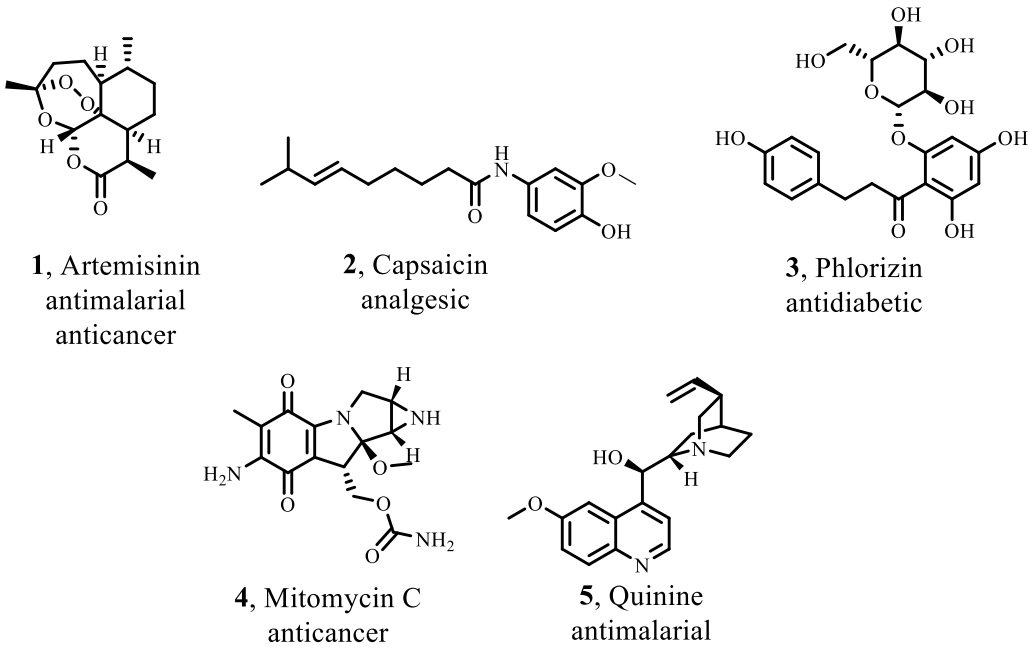


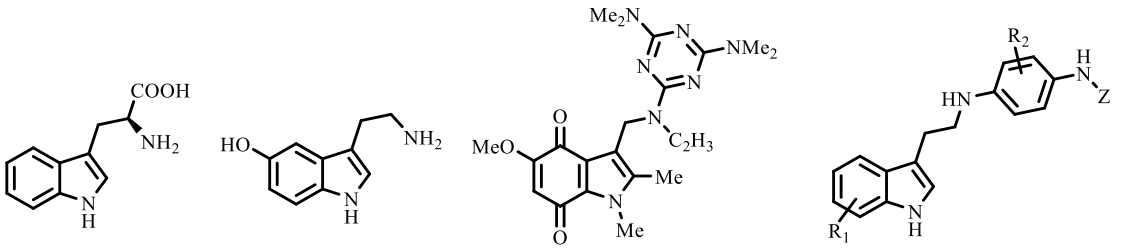
Fig. 1.1 – Chemical structures of natural products with different biological targets (1-5).

Usually, the isolated NPs are not the drug to be used for treatment of a disease. However, they can be used as lead compounds for the development of analogues. Furthermore, NP are selective to bind biological targets, becoming privileged structures as templates for the synthesis of biologically active NP-based molecules.[4] Indoles and spirooxindoles are scaffolds often found in NP. Dragmacidin E, jasplakinolide, elacomine and alstonisine are examples of these scaffolds.[6, 7]

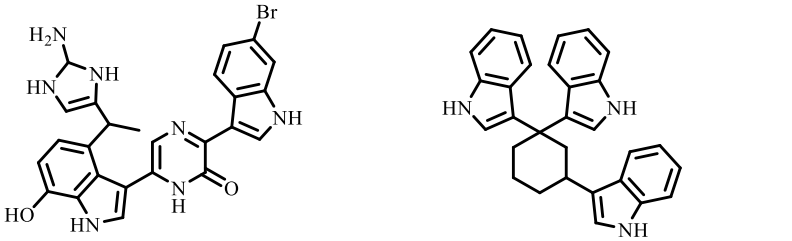
1.1. Indole derivatives

Indole based derivatives occur usually in natural products from plants, animals and marine sources. Tryptophan (**6**), for example, is an essential amino acid that is included in several biological processes. Serotonin (**7**) is a neurotransmitter and is derived from tryptophan. The indole core is known as a "privileged scaffold" in medicinal chemistry, because of its features and biological processes that are involved. Indole derivatives mimic the structure of peptides and are able to bind reversibly to enzymes.[2] Indoles have been reported to induce apoptosis in breast, squamous cell carcinoma, cholangiocarcinoma, colon, cervical, ovarian, pancreatic and prostate cancer cells.[8]

Pentamethylmelamine (PMM) is attached to an indole-2,3-dione moiety **8**. It entered in clinical trials in the 1970s for the treatment of ovarian carcinoma, but it was abandoned because of the lack of solubility in water. An indole derivative family **9** was patented in 2011 and it was reported for the treatment of several cancers (**Fig. 1.2**).[8] Dagmacidin D (**10**) has two indole groups bonded to a piperazine ring. It shows *in vitro* cytotoxicity with IC_{50} values of 15 µg/mL against P-388 cell lines (leukemia) and 1-10 µg/mL against A-549 (human lung), HCT-8 (human colon) and MDA-MB (human breast) cancer cell lines. The 1,1,3-tri(3-indolyl)cyclohexane (**11**) inhibits cancer cell growth in lung cancer cells, having positive pharmacologic properties. However, it triggers DNA damage and leads to the production of reactive oxygen species.[9]



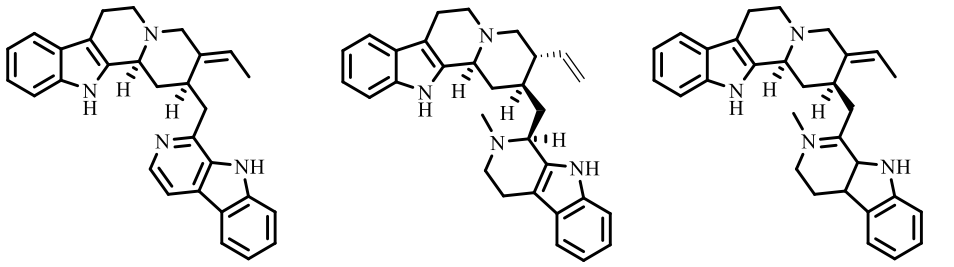
6, Tryptophan **7**, Serotonin **8**, Indole-PMM **9**, general structure protected by patent



10, Dragmacidin D **11,** 1,1,3-tri(3-indolyl)cyclohexane

Fig. 1.2 – Natural products and drugs with indole-derivative scaffolds 6-11.

The indole scaffold is also known for its antiparasitic activity. For example, usambarensine (**12**) and usambarine (**13**) are indole alkaloids, which are isolated from *Entamoeba histolytica.* These natural products have antiplasmodial activity with IC_{50} values of 0.023 and 0.13 μM. A derivative from usambarine, dihydrousambarensine (**14**), was tested against multidrug-resistant *P. falciparum* strain, K_1, showing to be 5 times more potent than chloroquine, the most used drug against this strain (**Fig. 1.3**). However, this last one showed to be inactive *in vivo* in mice affected with *P. berghei* chloroquine-sensitive strain. This means that dihydrousambarensine is selective to chloroquine-resistant strain.[10]



12, Usambarensine **13**, Usambarine **14**, Dihydrousambarensine

Fig. 1.3 – Indole derivatives with antiplasmodial activity.

Overall, among all natural products, indole alkaloids family is a very privileged scaffold, having very interesting anticancer and antimalarial activities.[10]

1.1.1. Spirooxindole natural products

Based on tryptamine, spirooxindole alkaloids **15-19** belong to a family that was first isolated from plants of the *Paocynaceae* and *Rubiaceae*. The main feature of this family is the spiro carbon in the position 3 of the oxindole core which gives several degrees of substitution around both rings linked to the spiro carbon. This carbon possesses a tetrahedral geometry, so the two rings can be almost perpendicular to each other, which gives unique conformational characteristics (**Fig. 1.4**).[11]

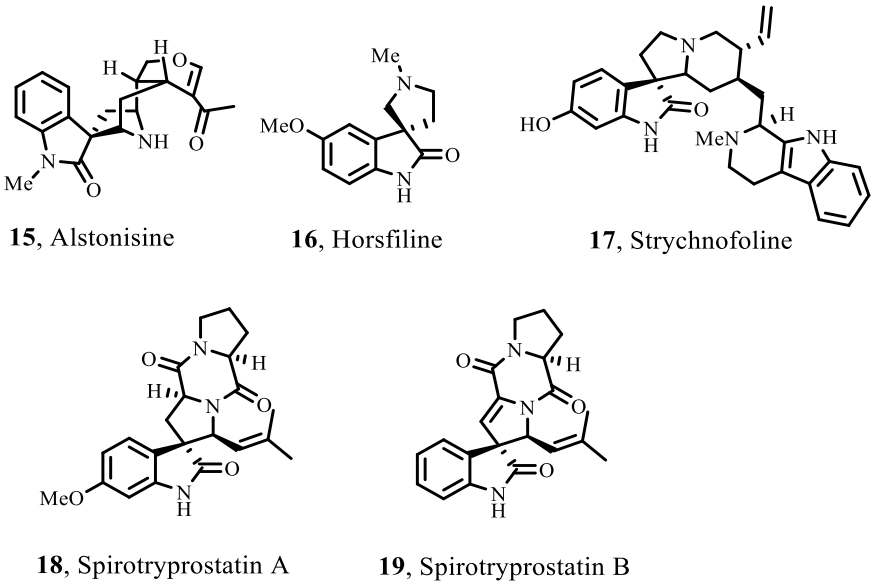


Fig. 1.4 – Spirooxindole natural products 15-19.

Several new drugs with different biological targets have been synthesized taking spirooxindole natural products as lead compounds (**Fig. 1.5**).

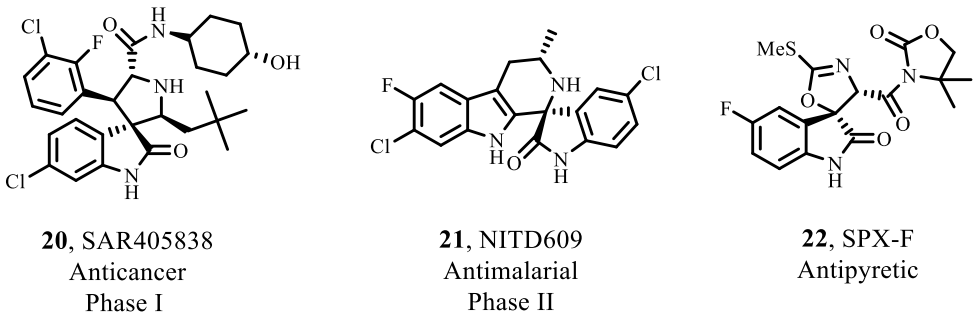


Fig. 1.5 – Drugs inspired in spirooxindole natural products.

1.2. Previous work in our research group and scope of the thesis

In our research group, we have been studying new indole-based scaffolds to reactivate p53 function. Different analogues of pyrrolidinyl-spirooxindole natural product, containing other 5-membered rings were developed.

The first family developed was the spiroisoxazoline oxindoles **23**. Eighteen new spiroisoxazoline oxindole were synthesized by reaction of hydroximoyl chlorides and 3-methyleneindoline-2,3-diones. This synthesis requires several steps, including the preparation of the dipolarophile. This family was tested in colon cancer cell lines, HCT116 and SW620. From them, one compound showed to have a GI_{50} value of 26.56 μM in HCT116 $p53^{(+/+)}$. They showed that any substituent in the aromatic rings increased the potency and the chlorine in the 6th position of the indole is important for the interaction with MDM2, corroborating with the co-crystal structure that was previously published. Furthermore, this atom occupies an unoccupied small pocket by indole and mimics the side chain of Trp23 of p53.[12]

Further optimization of spiroisoxazoline oxindoles led to the spiropyrazoline oxindole scaffold **24** by changing the isoxazoline oxygen to a *N*-phenyl group, resulting in an extra substituent. This family showed a high anti-proliferative activity in breast cancer cell lines, MCF-7 and MDA-MB-231, with GI_{50} values around 7.0 μM for MCF-7, showing greater selectivity for this cell line. The use of bromine atom in this scaffold showed to increase the potency as anticancer agent. Spiropyrazoline oxindole **24b** was also tested in human colon cancer cell line HCT116 to determine the DNA cell content. The experiment showed that most of cells were accumulated in G1 phase after 24h. Spiropyrazoline oxindoles showed to induce apoptosis.[13-15]

Taking in considerations that position 5 and 6 of the oxindole ring increase anticancer potency, new optimizations led to the spirooxadiazoline oxindoles **25**. Sixteen new spirooxadiazoline oxindoles with an [1,2,4]-oxadiazole ring were synthesized by reacting 3-imino-indolin-2-ones with nitrile oxides. In this family, the different positions of the halogens used as substituents were tested, including the 5th and the 6th positions of the oxindole and the *orto*, *meta* and *para* positions of the aromatic rings. This family was tested in human colon cancer cell lines, HCT116 and SW620. The most active compounds have both aromatic rings substituted in *meta* and *para* positions, with 8.5-fold and 4.3-fold increase in potency when compared to the non-halogens substituents. Spirooxadiazoline oxindole **25b** showed to inhibit p53-MDM2 interaction in the same extent as nutlin-3 at 10 and 20 μM. This same derivative also showed to be responsible for the accumulation of cancer cells in the cell cycle G1 phase.[16]

The family of spirooxadiazoline oxindoles **26** is a regioisomer of the family of spirooxadiazoline oxindoles **25**. The biggest difference between both families is the position of the heteroatoms in the oxadiazole ring, resulting in spatial orientation of the substituents. Damla Uyar, in her Master thesis, synthetized a small library of twelve spirooxadiazoline oxindoles **26**

and tested its anti-proliferative activity in human colon cancer cell lines. Spirooxadiazoline oxindoles **26b** showed to have more selectivity for SW620 cancer cell line, which has p53 with R273H mutation (**Fig. 1.6**).[17, 18]

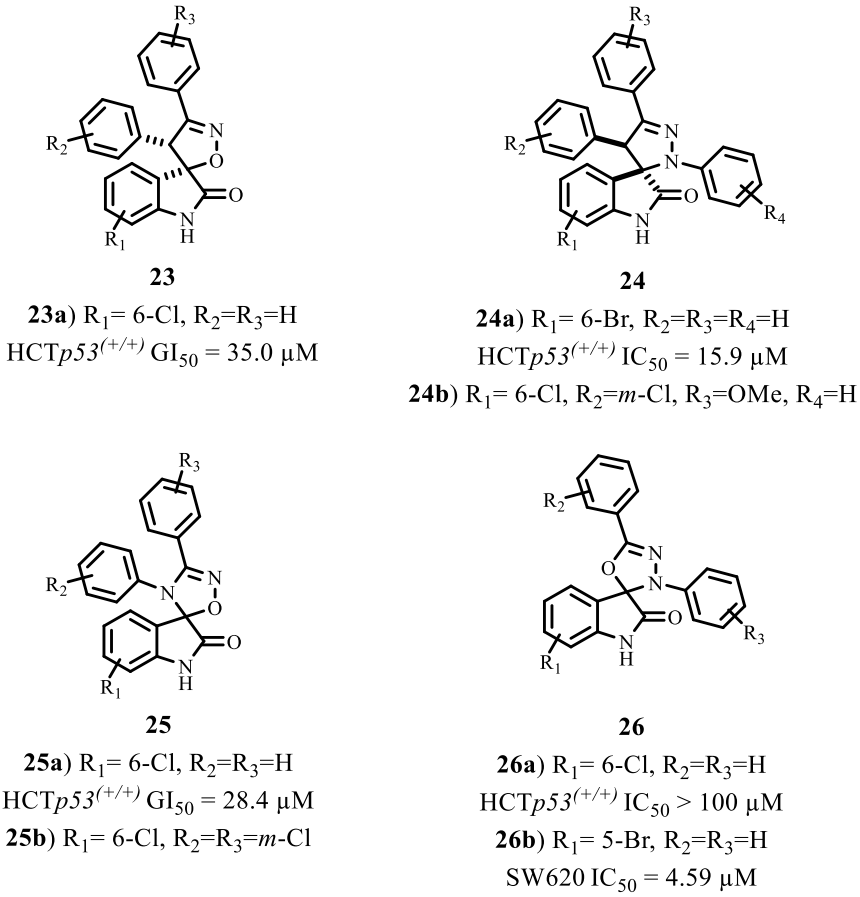


Fig. 1.6 – Optimization of spirooxindole scaffold by our research group.

In this thesis, the main goal was the development of a new library of spirooxadiazoline oxindoles with a [1,3,4]-oxadiazole ring, instead of an [1,2,4]-oxadiazole core to be tested in human breast adenocarcinoma cell lines, MCF-7 and MDA-MB-231, and *P. falciparum* chloroquine-resistant and sensitive strains (**Fig. 1.7**).

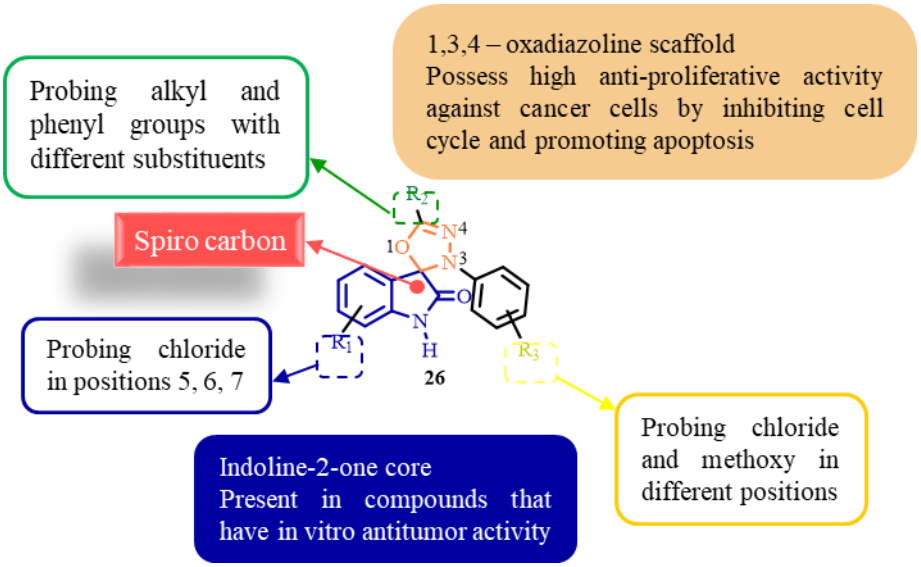
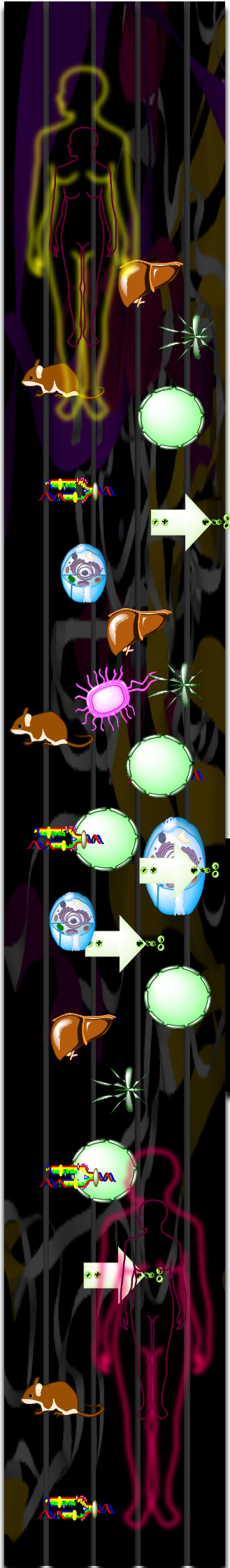


Fig. 1.7 – Design of the new spirooxadiazoline oxindoles family.



Chapter 2

SYNTHESIS OF SPIROOXADIAZOLINE OXINDOLES

Chapter 2. Synthesis of spirooxadiazoline oxindoles

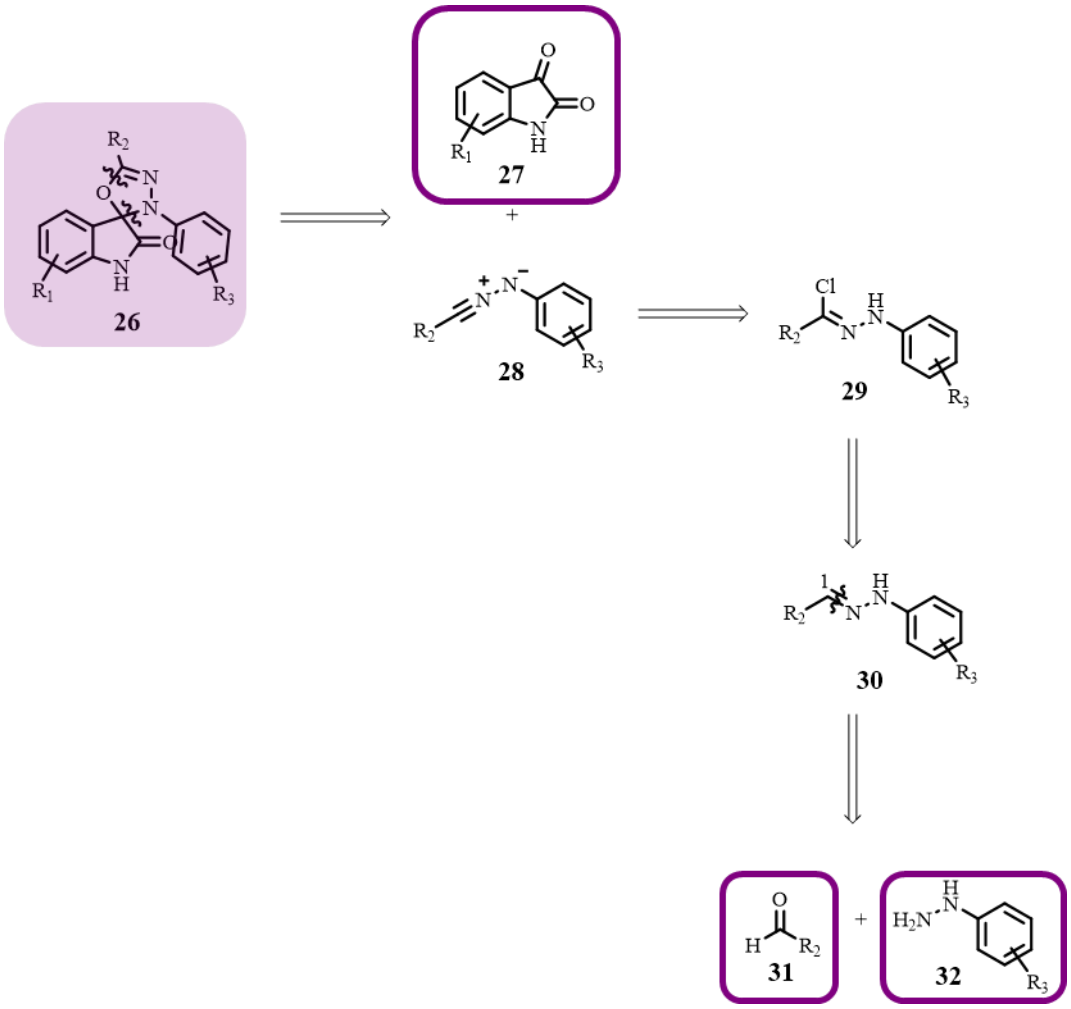
2.1. Introduction

The anticancer activity of the previous chemical families synthetized by our group has increased the interest in developing new spirooxindoles with five-membered ring.[12, 13, 16] As previously reported, the spirooxadiazoline oxindole family with an [1,2,4]-oxadiazole core has shown promising results in colon cancer cell lines, HCT116 and SW620 with *wt* p53 and *mut* p53, respectively.[16] Besides anticancer activity, oxadiazole and oxindole derivatives were described to have biological activity against therapeutically targets involved in other diseases.[19-22] For these reasons, joining these two scaffolds can lead to compounds with very interesting biological activities.

Based on all the results obtained by our research group, it was synthesized a library of twenty new spirooxadiazolines oxindoles with [1,3,4]-oxadiazole core.[12, 13, 15, 16, 23] The compounds were synthesized by 1,3-dipolar cycloaddition of isatin derivatives and nitrile imines (formed *in situ* from the corresponding hydrazonyl chlorides) derivatives, according to the **Scheme 2.1**.

The biggest advantage of these synthetic route is the commercial availability of the starting materials. The hydrazones **30**, that are chlorinated to afford the hydrazonyl chlorides **29**, are obtained by the reaction of aldehyde **31** and hydrazine **32** derivatives, which are commercially available. Several isatin derivatives **27** are also available, which can reduce several synthetic steps for the preparation of the dipolarophiles. In this library of spirooxadiazoline oxindoles **26**, only electron donating groups were tested. Besides, the substituents were tested in several positions: 5^{th}, 6^{th} and 7^{th} positions of the oxindole ring, *meta* and *para*-substituted phenyl ring and alkyl group as R_2 and *meta* and *para* substitution as R_3.

The anti-proliferative assays in human breast adenocarcinoma cell lines and a screening in *P. falciparum* chloroquine-resistant and sensitive strains were later performed.



Scheme 2.1 – Retrosynthetic route to obtain spirooxadiazoline oxindoles derivatives, highlighting the commercial available compounds.

Hydrazones were synthetized by condensing aldehyde **31** and hydrazine **32** derivatives in EtOH 20% for approximately 3 h (**Table 2.1**). This reaction involves the nucleophilic attack of the lone pair of electrons from the terminal nitrogen atom of the hydrazine to the carbonyl carbon of aldehyde with elimination of water. The precipitation of the product and its easy filtration are advantages in this procedure, giving very high yields (55-95%).[24]

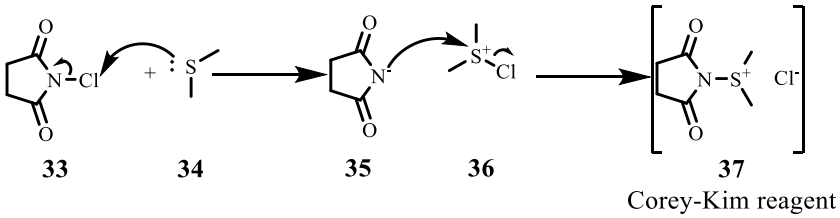
Table 2.1- Synthesis of hydrazones 30.

Compd	R_2	R_3	Yield/%
30a	H	*m*-Cl	95
30b	*p*-Cl	H	95
30c	H	*o*-Cl	56
30d	*p*-OMe	H	96
30e	*m*-Cl	*m*-Cl	98
30f	*m*-Cl	H	55
30g	H	H	92

The hydrazones **30a-g** were characterized by ^{1}H NMR. In all spectra, a deshielded singlet appears around 9.0 ppm corresponding to the hydrogen of the hydrazone group. In addition, the aromatic protons of the aromatic phenyl rings appear between 8.0 and 6.5 ppm, together with the proton of carbon 1 (assigned in **Scheme 2.1**). All NMR spectra were in accordance with the ones reported in the literature.[23, 25-28]

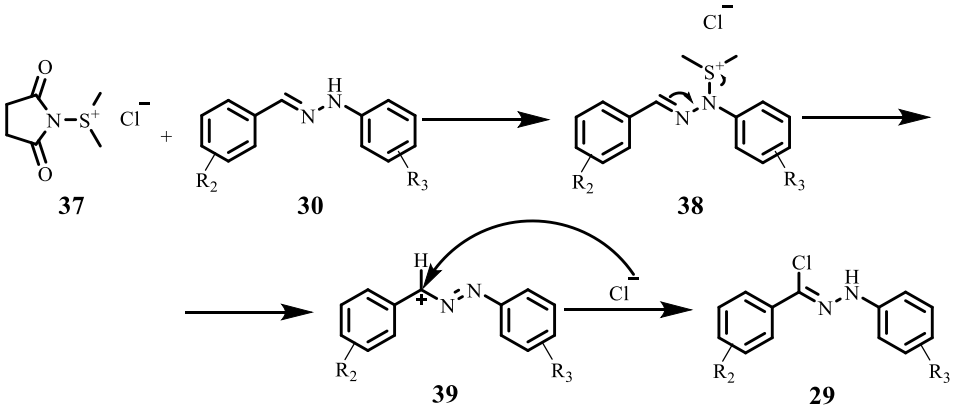
The second step of the synthetic route was the chlorination of the hydrazones **30** (**Scheme. 2.1**).

Hydrazonyl chlorides have been used for the synthesis of 5-membered rings (e. g. spirotriazoline oxindoles and spiropyrazoline oxindoles).[12-14] In order to substitute the hydrogen by a chlorine atom, *N*-chlorosuccinimide (**33**) and dimethyl sulfide (**34**) form the Corey-Kim reagent **37** (**Scheme 2.2**). This complex is also used for the oxidation of alcohols to aldehydes and ketones. It is a good alternative to the Swern oxidation, since it can be used in temperatures above -25ºC.



Scheme 2.2 – Formation of the Corey-Kim reagent.

The mechanism of the reaction is shown in **Scheme 2.3**.



Scheme 2.3 – Mechanism proposed by *Patel et al.* for halogenation of hydrazones.[29]

This mechanism involves the formation of a benzylic cation **39** which is stabilized by the adjacent nitrogen atom. Then, the cation undergoes nucleophilic attack by the counteranion Cl^- to give a phenylazobenzyl chloride, which tautomerizes to compound **29**.[29]

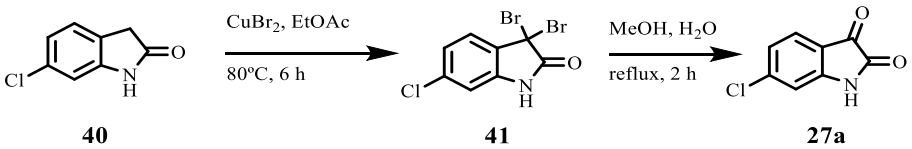
This reaction occurs in very low temperature (-78ºC), using dichloromethane as solvent with moderate to excellent yields (49-92%) (**Table 2.2**).

Table 2.2 – Synthesis of hydrazonyl chlorides.

Compd	R_2	R_3	Yield/%
29a	H	*m*-Cl	92
29b	*p*-Cl	H	72
29c	H	*o*-Cl	85
29d	*p*-OMe	H	49
29e	*m*-Cl	*m*-Cl	53
29f	*m*-Cl	H	70
29g	H	H	92

The ^{1}H NMR spectra of the hydrazonyl chlorides **29a-g** are very similar to the ones obtained for the corresponding hydrazones **30a-g**. However, since the hydrogen was replaced by a chloride, all protons are slightly less shielded. The biggest difference is the singlet of the proton of the hydrazone that appears around 10.0 ppm. All spectra are in accordance with the ones reported in the literature.[23, 29, 30]

An additional step was made for the synthesis of the 6-chloroindoline-2,3-dione (**27a**) from the 6-chlorooxindole. This compound was synthetized because of the high availability of the corresponding oxindole. This is a two-step reaction. First occurs the bromation of the position 3 of the indole derivative and then the oxidation of this position (**Scheme 2.4**).



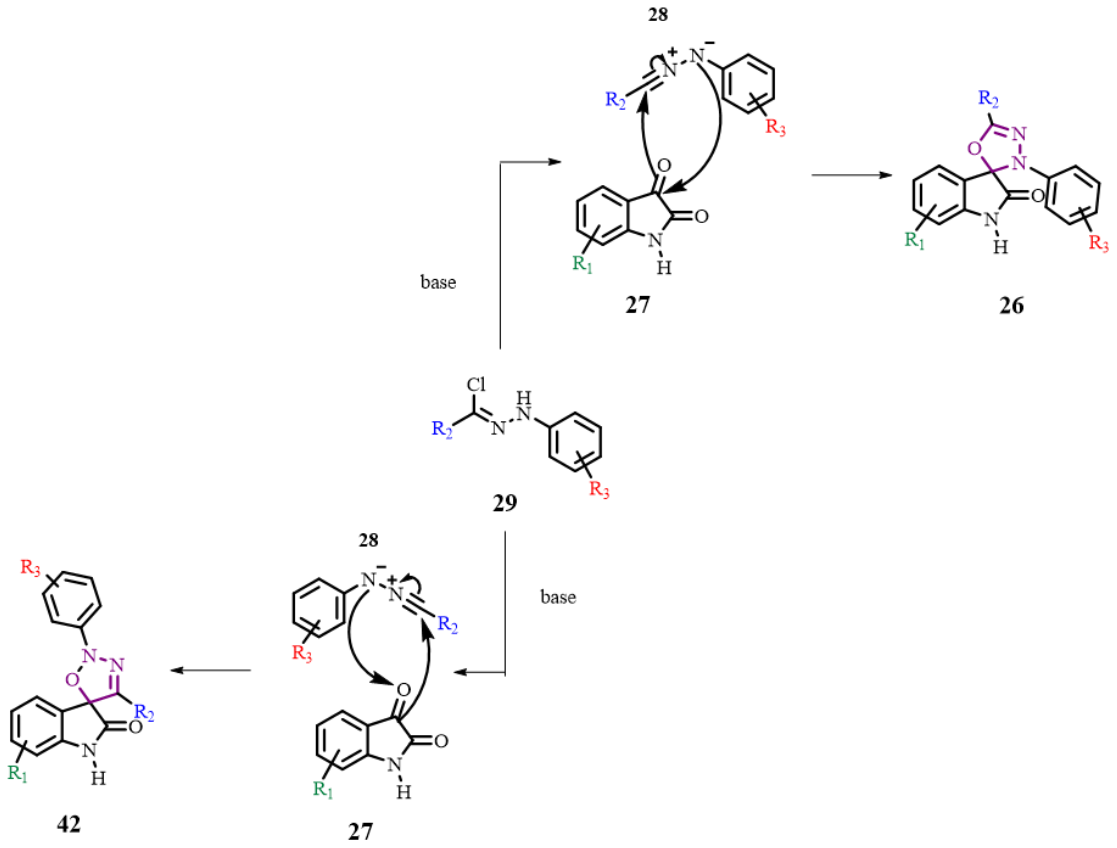
Scheme 2.4 – Synthesis of 6-chloroisatin (27a).

6-chloroisatin (**27a**) was obtained with a yield of 46% and the chemical structure was confirmed by ^{1}H NMR and it is in accordance with reported literature.[31]

2.2. Synthesis of spiro[indoline-3,2'-[1,3,4]oxadiazoline]-2-ones

2.2.1. Traditional synthetic route

The spirooxadiazoline oxindoles library was formed by reacting isatin and nitrile imines derivatives (formed *in situ* by the dichlorination of the corresponding hydrazonyl chloride) (**Scheme 2.5** and **Table 2.3**). To the date, the 1,3-dipolar cycloadditions of hydrazonyl chlorides with 3-imino-indolinones and 3-methylene indolinones are the most common. In this thesis, it is reported for the first the reaction of hydrazonyl chlorides with isatin derivatives. The biggest advantage of the use of isatin derivatives is their commercial availability, so the number of reaction steps is reduced.



Scheme 2.5 – 1,3-dipolar cycloaddition of isatin derivatives 27 as dipolarophiles and nitrile imines 28 as dipoles.

In this reaction, two regioisomers can be formed. They can be distinguished by NMR. For the regioisomer **26**, the spiro carbon and the C=N bond have their signals around 96.0 and 150.0 ppm in the ^{13}C NMR, respectively[17]. For the regioisomer **42**, since it has a different chemical environment, the signal of the spiro carbon should appear much more shielded, around 88.0 ppm.[32] The spiro carbon of the regioisomer **26** is bonded to two heteroatoms (oxygen and nitrogen) and two carbons, while the spiro carbon of the regioisomer **42** is bonded to an oxygen and three carbons. (**Fig. 2.2**)

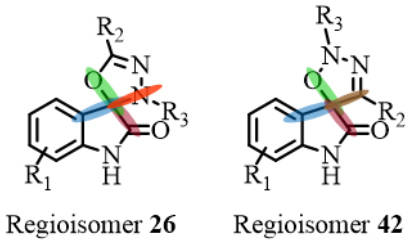


Fig. 2.1 – Difference in the spiro carbon for both regioisomers. In regioisomer 26, the spiro carbon is linked to a nitrogen (orange) and in regioisomer 42, the spiro carbon is linked to another carbon (brown).

.

Table 2.3 - Library of spirooxadiazoline oxindoles 26a-u and respective yields.

Cmpd	R_1	R_2	R_3	Yield /%	Cmpd	R_1	R_2	R_3	Yield /%
26c	5-Br	Ph	3-Cl	64	**26n**	5-Cl	Ph	3-Cl	64
26d	5-Br	3-ClPh	H	61	**26o**	7-Cl	Ph	3-Cl	68
26e	5-Cl	3-ClPh	H	85	**26p**	7-Cl	4-ClPh	H	67
26f	5-Br	4-ClPh	H	51	**26q**	H	Ph	3-Cl	87
26g	7-Cl	3-ClPh	H	54	**26r**	5-Cl	4-OMePh	H	63
26h	5-Br	4-OMePh	H	61	**26s**	7-Cl	4-OMePh	H	53
26i	H	Ph	4-Cl	68	**26t**	H	4-OMePh	H	42
26j	7-Cl	3-ClPh	3-Cl	67	**26u**	5-Br	t-Bu	H	90
26k	H	3-ClPh	H	80	**26v**	6-Cl	t-Bu	H	86
26l	7-Cl	Ph	4-Cl	68	**26w**	5-Br	H	H	67
26m	5-Cl	4-ClPh	H	64					

The spirooxadiazoline oxindoles **26a-x** were obtained with moderate to excellent yields (42 to 90%). In most of the reactions, the starting materials were not consumed completely. This can be explained by the possibility of the nitrile imine to dimerize.[17]

Compounds **26a-v** were characterized by NMR, infra-red and mass spectrometry.

The ^{1}H NMR spectra showed an increase of aromatic protons relatively to the hydrazonyl chloride spectra. The proton of the NH from the oxindole moiety appears around 10.0 ppm.

For all reactions, only regioisomer **26** was formed (the spiro carbon signal appeared between 95.5 and 97.5 ppm) as observed by ^{13}C NMR of **26c** as example (**Fig. 2.2**).

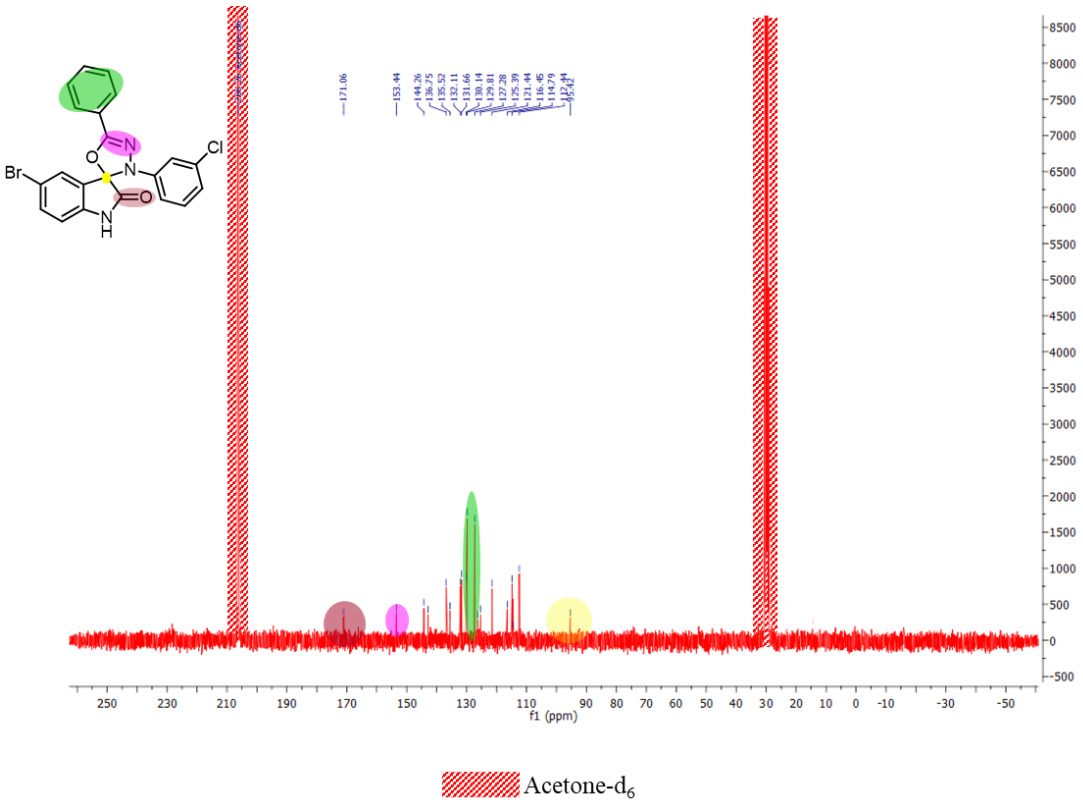


Fig. 2.2 – ^{13}C NMR of 26c. (yellow – spiro carbon, reddish brown – C=O, pink – C=N, green – equivalent carbons).

Another two signals are characteristic: the carbonyl carbon, and the carbon linked to the nitrogen by a double bond at around 170.0 and 152.0, respectively.[17]

IR spectra were obtained. Three main peaks were identified: the carbonyl stretch (C=O) around 1740 cm^{-1}, amine stretch (NH) around 3275 cm^{-1} and C=N stretch around 1600 cm^{-1}. Other peaks were identified as aromatic bonds. In the case of compounds **26u** and **26v,** an extra peak was identified at 2970 cm^{-1} corresponding to the alkyl C-H bond (**Fig. 2.3**).

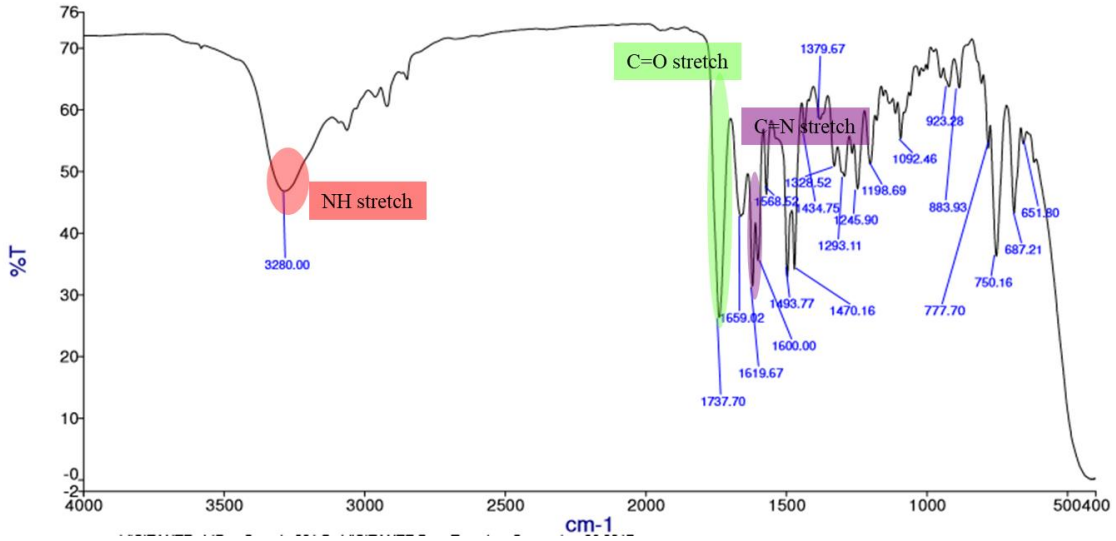


Fig. 2.3 – IR spectrum of 26i. Red, green and purple peaks are common to all family.

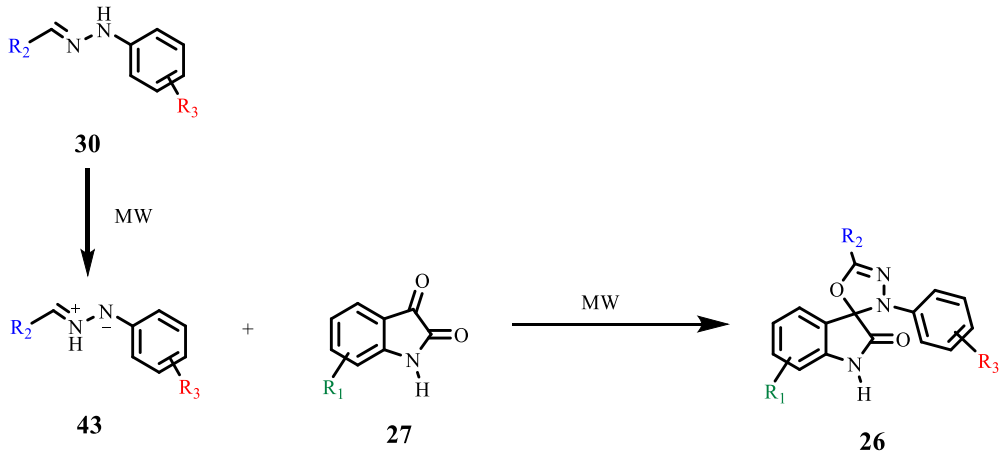
2.2.2. Microwave-assisted route

Microwave assisted synthesis is known to be a green and faster way to perform several reactions (**Table 2.4**), including the formation of 5-membered heterocycles.[33] For hydrazones, it has been reported some reactions for the formation of 5-membered rings from electron deficient dipolarophiles to form bipyrozolyl under microwave irradiation and solvent-free conditions.[34, 35] In this reaction, azomethine imines **43** are formed from the hydrazones **30** (instead of nitrile imines) and posterior 1,3-dipolar cycloaddition by reacting the azomethine imine **43** with the dipolarophile **27** (**Scheme 2.6**).

During the course of this thesis, microwave-assisted reactions to form spirooxadiazoline oxindole **26** derivatives were tried, in an attempt to synthetize for the first time spirooxindoles by MW, using also for the first time an isatin as dipolarophile.

Table 2.4 – Vantages and disadvantages of traditional and microwave assisted reactions.

Traditional	Microwave
• Room temperature reaction	• High temperature reaction
• Big scale reactions	• Small scale reactions
• No pressure	• Use of pressure
• Long time reaction	• Short time reaction
• Addition of TEA	• No need of base
• Use of dichloromethane as solvent	• Solvent free
• Synthetic route with 3 steps	• Elimination of chlorination step



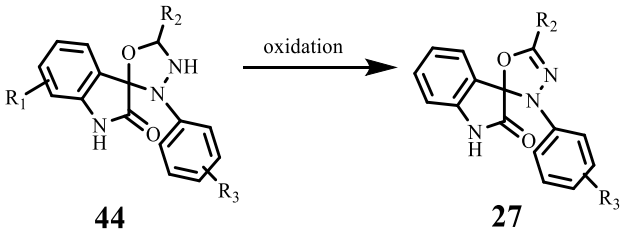
Scheme 2.6 –Synthesis of spirooxadiazoline oxindole 26 by microwave assisted reaction.

The reactions of the 5-bromoisatin with the hydrazones **30a-g** were tested under several different conditions: time (30 min to 2 h); temperature (90ºC to 180ºC); the use of solvent (dioxane) and free solvent reaction. Only two products **26f** and **26f** were synthetized by microwave assisted reaction (**Table 2.5**).

Table 2.5 – Spirooxadiazoline oxindoles synthesized by microwave assisted reaction

Cmpd	R_1	R_2	R_3	Traditional Yield /%	MW Yield /%
26d	5-Br	4-ClPh	H	61	71
26h	5-Br	4-OMe	H	51	79

It was already reported the 5-membered cycle formation from olefins, maintaining the same bond order. According to the mechanism described, the double bond is used for the formation of the ring as a normal 1,3-dipolar cycloaddition. However there is an autooxidation of the 5-membered ring with the oxygen that exists in the microwave vessel to form again the double bond.[36] In the case of this reaction, it is possible that before the rearrangement, the 5-membered ring **44** is too unstable and degrade in the system (**Scheme 2.7**). The stability of the hydrazonyl chlorides to high temperatures can also contribute to the degradation of the product, since the hydrazonyl chlorides used in this thesis have never been tested in microwave assisted reactions.[33-36]

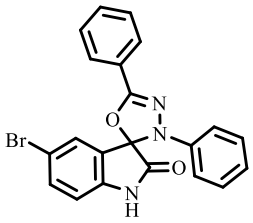


Scheme 2.7 – Oxidation that should occur in spirooxadiazoline oxindoles

Other conditions can be tested in the attempt to obtain the nitrile imines such as addition of NCS and triethylamine.[33]

2.3. Stability studies in NMR

The stability of the spirooxadiazoline oxindoles **26** were studied before the evaluation of biological activity to assure that compounds **26** do not degrade in solution during the biological assays. For this reason, in a NMR tube, spirooxadiazoline oxindole **26w** (**Fig. 2.4**) was dissolved in DMSO-d_6 (solvent used in biological assays) and kept at room temperature. The stability was performed using NMR spectroscopy, obtaining a new ^{1}H NMR of the solution every week (**Fig. 2.5**).



26y

Fig. 2.4 – Structure of 26w.

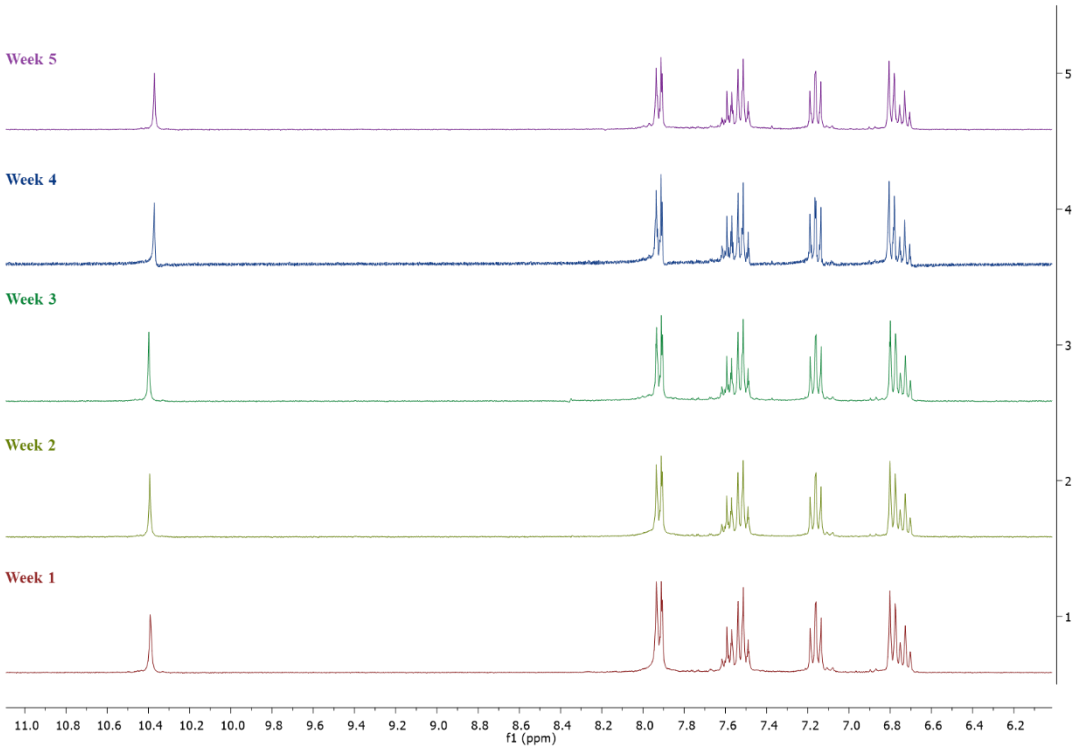
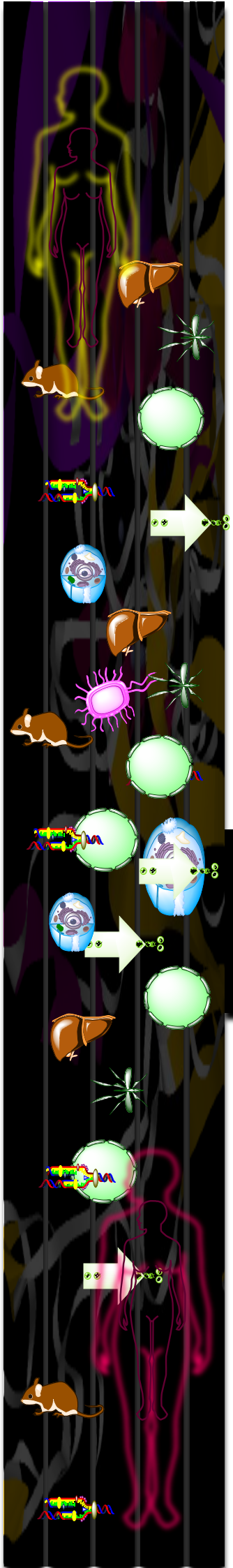


Fig. 2.5 – Spectra of 26w in DMSO-d_6 every seven days.

Comparing the five spectra, it is possible to conclude that compound **26w** is very stable in DMSO-d_6. Only after the 5th week, it was observed very small extra peaks in the spectrum.

Despite, these small changes, the compound does not suffer degradation in DMSO, so it can be safely used in biological assays.



Chapter 3

SMALL MOLECULES ACTING AS p53 REACTIVATORS

Chapter 3. Small molecules acting as p53 reactivators

3.1. Introduction

3.1.1. p53 protein

The p53, a short-lived protein, known as the guardian of the human genome, was discovered in 1979 as a cellular 53 kDa nuclear phosphoprotein bond to the large transforming antigen of the SV40 DNA virus.[37-39] The p53 gene (TP53) was first cloned in 1983.[40] Six years later, p53 was shown to block the transformation of rat embryo fibroblasts. In 1990, TP53 was identified in several Li-Fraumeni syndrome, a rare condition which entire families are affected by several types of cancers. In 1993, p53 was considered the molecule of the year.[40]

It belongs to a small family of related proteins which includes p63 and p73. While these two proteins have important roles in the normal development, p53 has a special role to prevent tumour development.[41] The wild type (*wt*) p53 protein binds to specific nucleotide sequences termed p53-responsive elements that, when together, stimulate a p53-dependent expression.[42] This tumour suppressor is regulated by degradation via the ubiquitin-proteasome pathway and monitors the integrity of the cells, and is one of the responsible to control cell cycle, DNA repair and synthesis, cell differentiation, genomic plasticity, senescence, angiogenesis and programmed cell death, including apoptosis and cell cycle arrest (**Fig. 3.1**).[37, 38, 43, 44]

High concentrations of p53 protein are found during the meiotic process of spermatogenesis. At that moment, the genome recombines and is responsible for genetic diversity. DNA damage in somatic cells caused by radiation or topoisomerase inhibitors leads to stabilization of *wt* p53 protein. p53 arrests cells in G1 phase of cell cycle, which is the moment when the DNA is repaired, avoiding mutations.[37]

The p53 pathway uses several mechanisms to arrest cell cycle progression, preventing the propagation of DNA damage and repairing it. If the damage is too severe, p53 leads to apoptotic cell death to prevent any malignant proliferation of damaged cells. Therefore p53 plays a protective role in normal cells by limiting the propagation of damaged cells.[43] Since this type of cell death requires a genetic program, mutations in apoptotic pathways produce drug resistant tumours. p53 has a main role in apoptosis induced by anticancer agents in damaged cells.[45] When p53 is mutated (*mut* p53), its function of tumour suppressor can be affected.

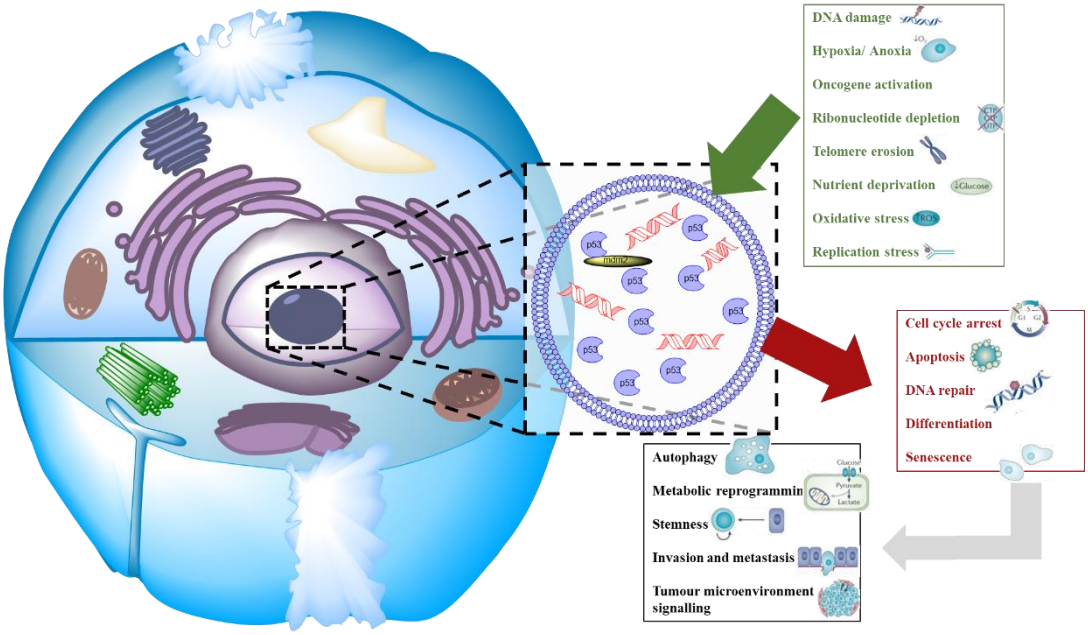


Fig. 3.1 – p53 regulates cellular response to stress. Several stress factors activate p53 and lead to a stabilization and accumulation of it in cell nuclei. Some genes are activated and trigger several responses. [Adapted from [43, 46]]

The p53 tumour suppressor gene is mutated or deleted in half of all types of cancer.[38, 40, 43, 47] When mutated, p53 protein has a different conformation, failing to bind DNA and regulate the transcription of p53 responsive genes. This phenomenon is known as loss of function mutation. *Wt* p53 is the most common in malignancies, such as acute myeloid leukaemia (AML) and melanoma. In these cases, cellular oncogene products of DNA tumour viruses bind to p53 protein and block its ability of transcription.[40, 47] Murine Double Minute 2 (MDM2) and Murine Double Minute X (MDMX) are two examples of these inhibitors.

Table 3.1 – Compounds that act in *wt* and *mut* p53.[48]

Molecule/ compound	Mechanism of action	Target	Stage of development
Nutlins RG7112	Inhibits p53-MDM2 interaction	MDM2	Phase I clinical trials
Benzodiazopinediones	Inhibits p53-MDM2 interaction	MDM2	Preclinical
Spirooxindoles	Inhibits p53-MDM2 interaction	MDM2	Phase I clinical trials
RITA	Inhibits p53 binding	*Wt* and *mut* p53	Preclinical
Serdemetan	Inhibits p53-MDM2 interaction	MDM2	Phase I clinical trials
SJ-172550	Inhibits p53-MDM2/X interaction	MDMX	Preclinical
RO-2443/RO-5693	Inhibits p53-MDM2/X interaction	MDMX	Preclinical
XI-011	Repression of MDMX promoter	MDMX	Preclinical

3.1.2. p53-MDM2 inhibition

MDM2 is a phosphoprotein of 54 kDa that has a putative nuclear localization signal and two zinc fingers. The MDM2 gene is located on chromosome 12q13-14 and encodes for 491 amino acid protein.[39] It can be associated with *mut* and *wt* p53 and its excess functions as a negative regulator of this protein.[40, 49] It is an E3 ubiquitin ligase that targets p53 degradation by the proteasome pathway.[40, 44]

Inactivation of the p53 tumour suppressor by mutation or overexpression of negative regulators occurs frequently in cancer. As explained before, p53 plays an important role in the regulation of proliferation and apoptosis in DNA damaging chemotherapies, so the reactivation of p53 by disruption of p53-MDM2 protein interaction is an interesting strategy for targeted anticancer therapy.[38, 47]

According to the X-ray structure, the p53-MDM2 edge bears a total of 1498 Å of surface area. The interface has a steric complementarity between the MDM2 cleft and the hydrophobic face of α-helix of p53, which includes Phe19, Leu22, Trp23 and Leu26. These four amino acids make a

sequential and extensive van der Waals interaction with the MDM2 pocket. They are complemented with two intermolecular hydrogen bonds: the interaction of the Phe19 backbone amide of p53 with the Gln72 side-chain of MDM2 and the nitrogen of p53 of the Trp23 indole group with the Leu54 backbone carbonyl moiety of MDM2 (**Fig. 3.2**).[38, 40]

The core of p53 is a region which can interact with DNA in a sequence specific-manner. Usually the mutations observed in tumours occur in regions where critical residues of DNA contact or their conformation are changed. For optimal binding to DNA, the protein is in a tetrameric state since there are four interactions of four separate p53 molecules via the tetramerization domain. The C-terminal region is responsible for regulatory properties and has mainly basic residues. This region suffers modifications by acetylation, phosphorylation, *O*-glycosylation and RNA binding. The DNA binding domain is separated from the transcriptional domain by several proline residues which can interact with signal transduction molecules that have SH^3 binding domain. On the other side, the acidic N-terminal domain activates the expression of target genes while giving the support for transcribing the new mRNA. This region is also important for stability and activity of the interactions of p53 and other proteins as MDM2, forming specific complexes, which allows targeting of ubiquitin-mediated proteolytic machinery.[49, 50] Modifications by phosphorylation in this region change the properties of this area, including its conformation, and as a consequence, the interaction of p53 with proteins.[50] According to the studies by Lane's laboratory, the disruption of p53-MDM2 interactions leads to an uncontrolled increase of p53, since MDM2 takes p53 from the nucleus to the cytoplasm, where it should be inaccessible to target DNA for transcription and it should be degraded by ubiquitination through its E3 ubiquitin ligase activity.[44, 51-53] MDM2 can also auto ubiquitinate itself, leading to self-degradation.[39]

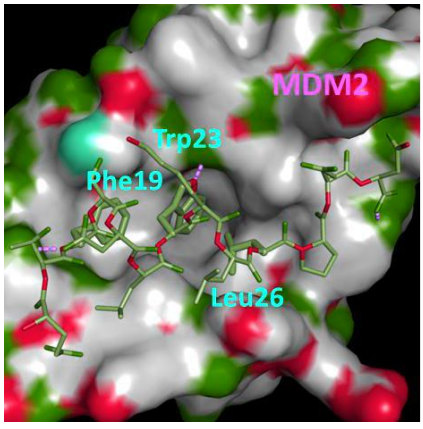


Fig. 3.2 – MDM2 (surface)-p53 complex (PDB ID: IYCR). The main interactions are made by Leu26, Phe19 and Trp23 from a small amphipathic p53 derived α-helix of p53 and the MDM2 pocket. [Adapted from [44]]

p53 is a transcription factor for MDM2 which results in an autoregulatory feedback loop, so p53 protein regulates the transcription of MDM2 gene and, in turn, MDM2 protein regulates the activity of p53 protein.[39, 40, 42-44]

The gene transcription by p53 is influenced by modifications such as phosphorylation or interactions with other proteins. MDM2 binds to p53 at its N-terminal domain and inhibits its transcriptional activity. Its amplification on chromosome 12q12-13 and excess of the gene product inactivates p53, so its tumour suppressor function is also shut down. Sequence-DNA binding and transcriptional activity of the *wt* p53 protein are also reduced in the presence of oncoproteins produced by some DNA viruses.[37, 43]

3.1.2.1. Small molecules inhibitors of p53-MDM2 interaction

MDM2 overexpression is oncogenic and is associated with the late stage disease, resistance to chemotherapy and radiotherapy and poor prognosis.[39] Furthermore, genetic studies have shown that loss of p53 activity induces tumour formation, while its activity restoration leads to the tumour regression.[40] p53 and MDM2 are both valuable targets for developing anticancer agents. One of the most popular strategies for reactivation of p53 activity is the use of non-peptidic small molecule inhibitors targeting the van der Waals protein-protein interactions site between MDM2 and p53 by mimicking the three critical amino acids residues from p53 pocket.[43, 54] The use of small molecules inhibitors for targeting p53-MDM2 and p53-MDMX interactions in a dual inhibition is another attractive strategy.[39, 40] Other approaches are blocking MDM2 expression, inhibiting the E3 ubiquitin ligase activity of MDM2 and targeting the protein-protein complex of proteins that interact with MDM2.[39]

Several study groups have shown the use of antisense oligonucleotides to inhibit MDM2 expression. They also showed that both *wt* p53 and *mut* p53 respond to MDM2 inhibition. These same groups have investigated the use of natural products such as curcumin, genistein and ginsenosides to down-regulate MDM2 expression.[39, 55-57]

Recently, small molecules were also reported to target E3 ligase activity of MDM2. They inhibit the ubiquitination of p53 *in vitro*, allowing the activation of p53 signalling, inducing cell cycle arrest and/or apoptosis. The first groups of these kind of inhibitors were the arylsulfonamide, bisaryluarea and acylimidazolone, first described in 2002. They allosteric inhibit MDM2 by blocking rearrangements of MDM2 that can be necessary for p53 ubiquitination.[39]

The most important families of inhibitors of p53-MDM2 interaction are cis-imidazolines **45** (or also known as nutlins), spirooxindoles **20**, piperidinones **48**, 1,4-diazepines and isoindolinones (**Fig. 3.3**).

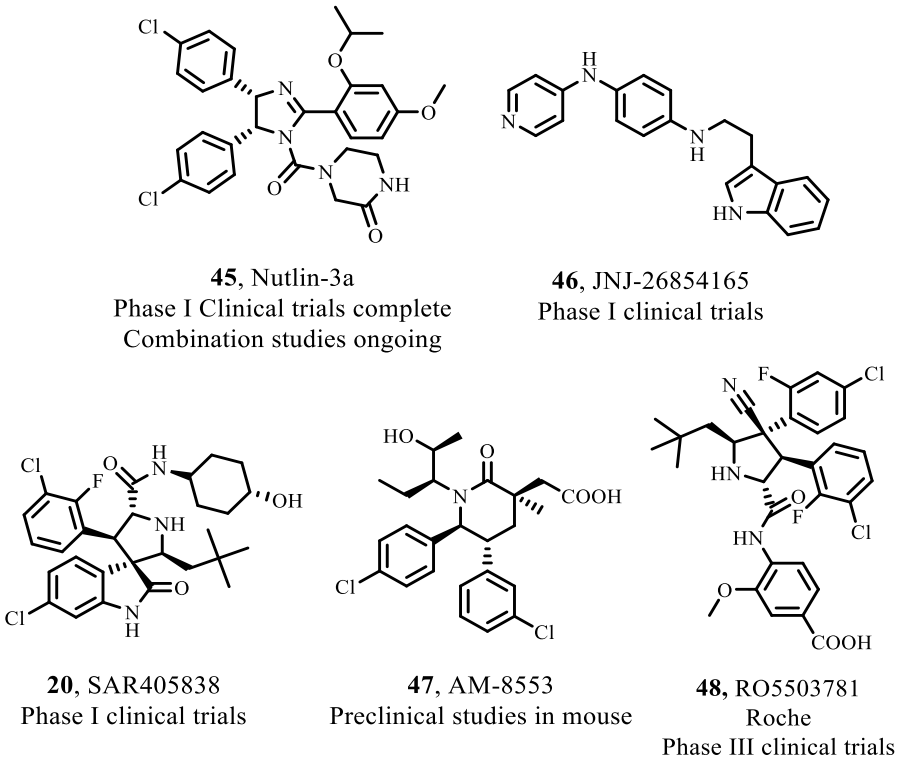


Fig. 3.3 – p53-MDM2 small molecule inhibitors that are in preclinical and clinical trials (compounds 20 and 45-47).[39]

3.1.3. p53-MDMX inhibition

MDMX is an additional MDM2 family member of which the 6-chlorotryptophan residue projects into the Trp23 binding site and optimizes the steric complementarity of p53-MDMX interface. It was discovered in 1996 as a close homolog protein of MDM2 that potentiates the ubiquitin ligase activity of MDM2 and blocks its p53 transcriptional activity without its degradation.[44] Like MDM2, it is overexpressed in several tumours. Due to its characteristics, it is important for targeting p53-MDMX interaction.

Despite being similar, MDM2 and MDMX have some conformational differences as well as some different amino acids which lead to a poorly inhibition by p53-MDM2 small molecules inhibitors.[38, 40] The critical residues for binding p53 are identical of MDM2, however the residues around Leu26 pocket are different, leading to different shape and size. These residues include Met53 (Leu54 in MDM2), Leu98 (Ile99 in MDM2), Leu102 (Ile103 in MDM2) and Pro95 (His96 in MDM2), resulting in a smaller binding groove in MDMX. Around the Trp23 pocket, the Phe86 residue of MDM2 is replaced by Leu85 residue in MDMX (**Fig. 3.4**).[44] Both have an acidic N-terminal p53 binding domain, C-terminal ring domain and a zinc finger domain.[44] The ring-finger domain located at the C-terminal end of both proteins is a well-conserved region. The integrity of this region is required for heterodimerization. The C_4 zinc finger is also conserved in both proteins. MDMX was shown to be a cytoplasmic protein and it is dependent on other

proteins, such as MDM2, for nuclear localization.[38-40] Disrupting the MDM2/MDMX heterocomplex inhibits the activity of p53 and cannot be reversed. Because of their critical and non-redundant role in p53 inhibition, targeting both MDM2 and MDMX can fully activate *wt* p53 in tumour cells.[44]

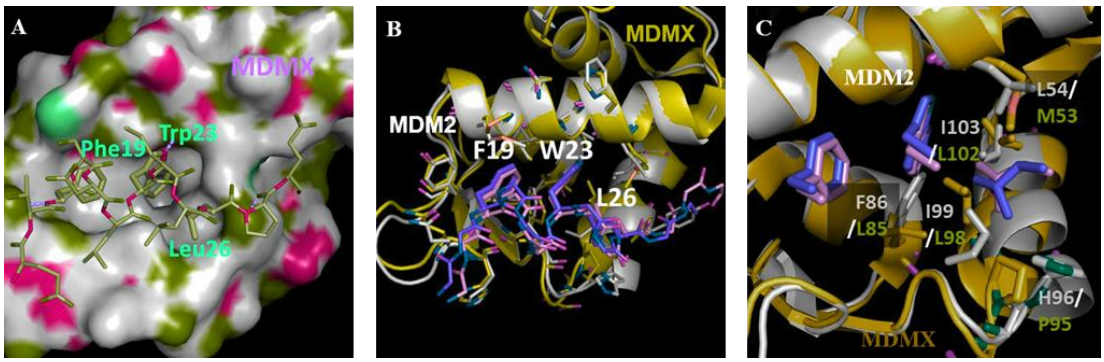


Fig. 3.4 – A) MDMX (surface)-p53 complex (PDB ID: 3DAB). The main interactions are made with Leu26, Phe19 and Trp23 from a small amphipathic p53 derived α-helix of p53 and the MDMX pocket. B) Superimposition of the two complexes. MDM2 is in white, MDMX is in yellow and p53 are in pink and blue, respectively. C) Superimposition of the two complexes. The residues that are non-identical are identified. [Adapted from [44]]

Because of the differences of the binding site of MDM2 and MDMX, inhibitors of p53-MDM2 interactions have low affinity for MDMX. For this reason, the search of MDMX antagonists is mandatory for a more robust p53 activation.

SJ-172550 was the first molecule to be identified to kill cells by reversibly bind to MDMX. Two compounds of a series of indolyl hydantoyn compounds, RO-2443 and RO-5693, showed also to bind to the MDMX pocket, being then described as potent inhibitors of MDMX. Another compound, XI-011 was reported to activate p53 in breast cancer cells, by inhibiting its interaction with MDMX. This inhibition is through the transcriptional repression of MDMX promoter. All these compounds are still in a preclinical stage.[48]

3.1.4. Spirooxindole core

In 2005, the Wang group at the University of Michigan reported the structure-based design of spirooxindole as a new class of potent small-molecule inhibitors of p53-MDM2 interaction. The oxindole group was found to mimic the Trp23 moiety (**6**) and spirooxindole-containing natural products **49** were identified and docked to MDM2 protein (**Fig. 3.5**).

MI-5 (**51**) was designed to interact effectively with MDM2. The oxindole moiety inserts into the Trp23 pocket, the phenyl group occupies the Phe19 pocket and the isopropyl group occupies the Leu26 pocket. The substitution of isopropyl group to *t*-butyl group and the introduction of a chlorine atom in position *meta* in the phenyl group lead to MI-17 (**52**) which is 100-fold more potent than MI-5.

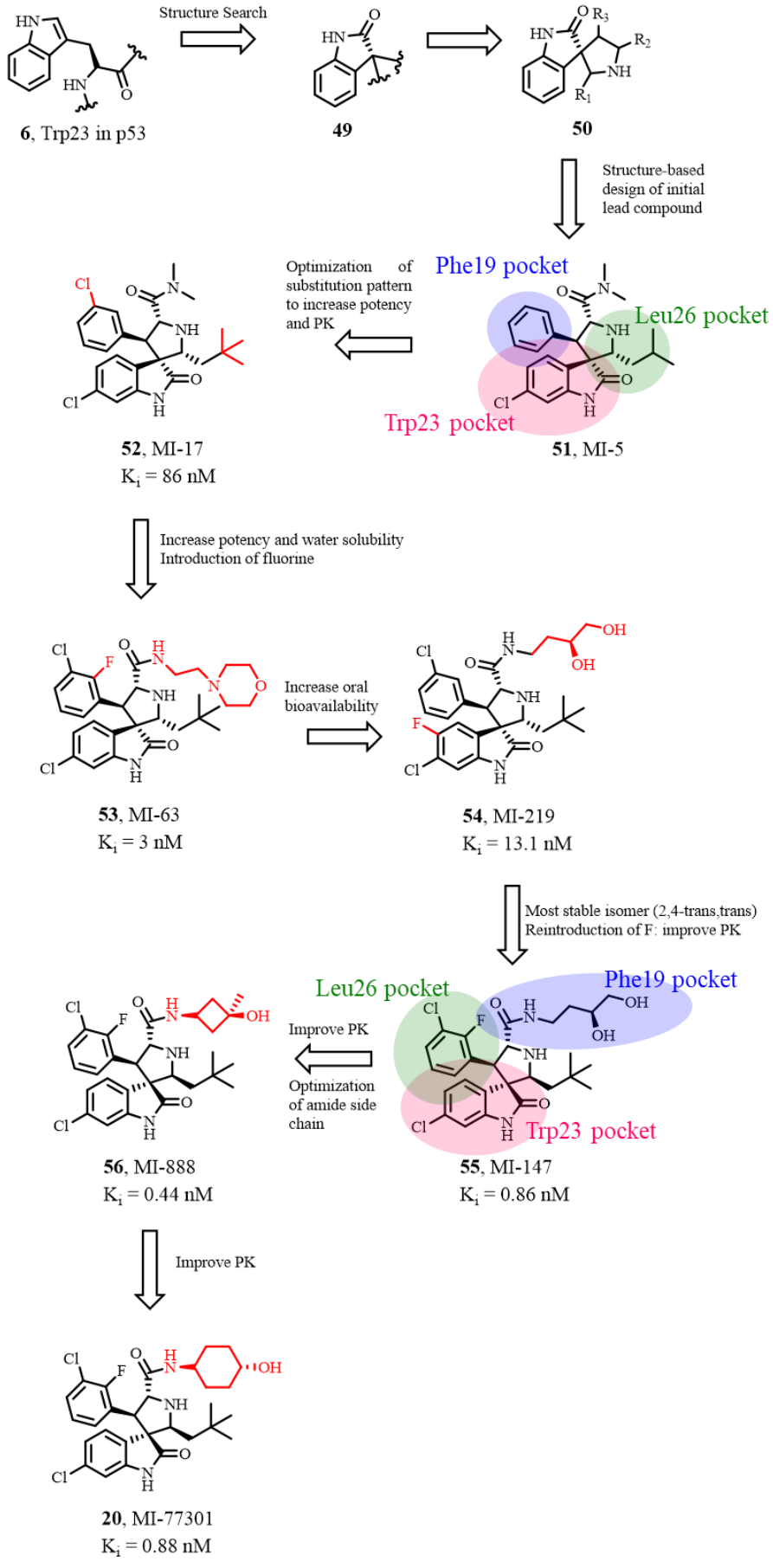


Fig. 3.5 – Spirooxindoles optimization to SAR405838 (or MI-77291) (20). [adapted from [44]]

Further optimizations were made leading to MI-63 (**53**). Despite having good water solubility, it has a very modest oral bioavailability. To overpass this problem, more modifications were made leading to MI-219 (**54**). This compound was proved to induce activation of p53 by blocking p53-MDM2 interaction in cells with *wt* p53. It was shown to inhibit cell growth in cancer cells and has more than 10-fold selectivity in tumour cells with mutated or deleted p53. Changing the stereochemistry, MI-888 (**56**) has a much higher affinity to MDM2, selectivity in inhibition of tumour cell growth in cancer cells with *wt* p53, oral bioavailability and it is capable of achieving total and durable tumour regression.[44]

Currently, SAR405838 (**20**), an analogue of MI-888, is in Phase I of clinical trials. It was developed by Sanofi-Aventis as a derivative of MI-219. It works as a MDM2 antagonist, binding to the p53 pocket of MDM2 molecule with specificity and selectivity, resulting in p53 reactivation at low nanomolar concentrations and induces cell-cycle arrest and apoptosis.[54, 58] Spirooxindole molecules were inspired by the presence of indoles in natural products. The starting point that led to SAR405838 was to mimic the three most important amino acids and their van der Waals and hydrogen bond interactions. The X-ray structure of MDM2 complexed with an spirooxindole analogue showed that the oxindole moiety inserts deeply into the Trp23 pocket of MDM2. The 2-fluoro-3-clorophenyl ring binds to the Leu26 binding site. Its rotative plane allows the halogens to reach the bottom of the MDM2 pocket. The Phe19 cleft is filled by the neopentyl side chain which requires an induced fit. For that, the backbone of His73 must retract outward from the pocket and allows the side chain of Tyr67 to rotate (**Fig. 3.6**).[38]

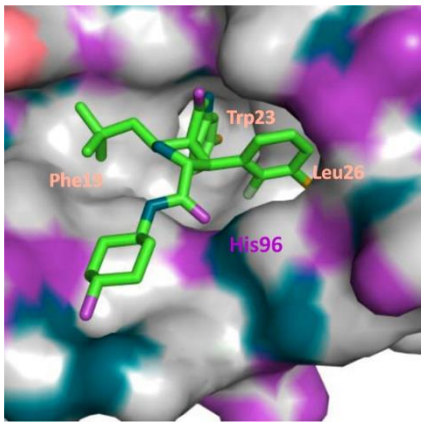


Fig. 3.6 – Co-crystal structure of MDM2 (surface) and SAR405838 (18) (green). [Adapted from [44, 59]]

3.2. Evaluation of anti-proliferative activity of spirooxadiazoline oxindoles **26** in breast cancer cells

Considering the results previous obtained by our group and the importance of spirooxindole derivatives in natural products and medicinal chemistry, the synthesized spirooxadiazoline oxindoles **26a-v** were tested against two breast cancer cell lines, MCF-7 and MDA-MB-231 (**Table 3.2**).[11, 14, 15, 60]

MDA-MB-231 is a triple negative breast cancer cell line which means it has lack of oestrogen, progesterone and ERBB2 receptors.[61] It has a basal like molecular subtype which has unique features as molecular profile and a set of risk factors like aggressive and early pattern of metastasis, limited treatment options and poor prognosis.[62] Furthermore, in this cell line, p53 has a R280K mutation. On the other hand, MCF-7 is a luminal type breast cancer cell line which oestrogen and progesterone and glucocorticoids receptors are present.[63] In this cell line, p53 is present as *wt* p53. Both cell lines are human breast adenocarcinomas.

According to the results, compounds **26n** and **26u** showed to inhibit around 50% of the cell growth at concentration of 50 μM, in both cell lines tested. Compound **26h** showed to be more selective to inhibit the cell growth, around 49%, in MCF-7 cell line. On the other hand, compounds **26c** and **26v** showed to be more selective to inhibit around 40% of cell growth in the *mut* p53 breast cell line.

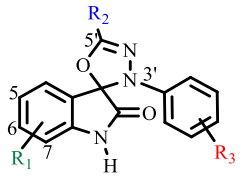
In this library of spirooxadiazoline oxindole, only electron donating groups, as halogens and methoxy group, were tested. The size of the R_2 substituent was tested by the replacement of an aryl group to an alkyl group. Furthermore, their *meta* and *para* positions at the aromatic rings were also tested. In the oxindole ring, the bromine and chlorine atoms were tested at positions 5, 6 and 7.

Compounds **26c**, **26h**, **26n**, **26u** and **26v** (**Fig. 3.7**) have bromine or chlorine at positions 5 and 6 of the oxindole ring. The derivatives with a substituent in position 7 or unsubstituted at the oxindole ring are not active for these two cell lines. The *m*-chlorophenyl ring at the position 3' in compounds **26c**, **26n**, **26u**, **26v** was found to be important for the inhibition of tumour cells growth.

Compound **26h** has a *p*-methoxyphenyl group as R_2, showing selectivity for inhibition of MCF-7 cell growth. Compounds **26r-t** also have this substituent. However, they do not show this selectivity. For this reason, the 5-bromine atom of the oxindole ring can be responsible for the difference in selectivity of compounds **26h** and compounds **26r-t**.

Comparing compounds **26c** and **26n** with compounds **26u-v**, the R_2 substituent does not show any improvement in activity by changing from an aromatic ring to an alkyl group.

3.2.1. Assessment of cell viability and SAR study



26

Table 3.2 – *In vitro* anti-proliferative activity at 50 µM.

Cmpd	R_1	R_2	R_3	%cell viability MCF-7	%cell viability MDA-MB-231	Cmpd	R_1	R_2	R_3	%cell viability MCF-7	%cell viability MDA-MB-231
26c	5-Br	Ph	*m*-Cl	56±19	38±8	**26m**	5-Cl	*p*-ClPh	H	100±3	75±8
26d	5-Br	*m*-ClPh	H	70±4	85±7	**26n**	5-Cl	Ph	*m*-Cl	52±9	58±5
26e	5-Cl	*m*-ClPh	H	98±2	78±6	**26o**	7-Cl	Ph	*m*-Cl	101±4	75±5
26f	5-Br	*p*-ClPh	H	103±6	78±1	**26p**	7-Cl	*p*-ClPh	H	98±4	89±0
26g	7-Cl	*m*-ClPh	H	98±6	91±13	**26q**	H	Ph	*m*-Cl	97±8	87±5
26h	5-Br	*p*-OMePh	H	49±9	106±12	**26r**	5-Cl	*p*-OMePh	H	74±15	64±10
26i	H	Ph	*p*-Cl	106±2	78±0	**26s**	7-Cl	*p*-OMePh	H	69±11	70±10
26j	7-Cl	*m*-ClPh	*m*-Cl	71±9	98±6	**26t**	H	*p*-OMePh	H	84±20	103±12
26k	H	*m*-ClPh	H	81±10	95±10	**26u**	5-Br	t-Bu	H	45±1	58±3
26l	7-Cl	Ph	*p*-Cl	98±1	85±5	**26v**	6-Cl	t-Bu	H	78±2	46±6

Cell viability was determined by MTT method after 48h. Each value is the mean (%±SD) of three experiments performed in triplicate.

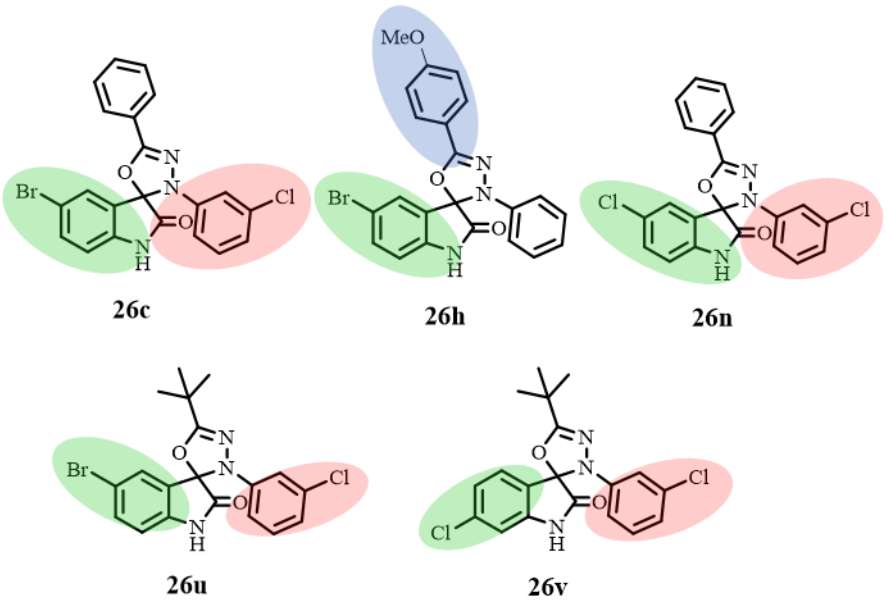


Fig. 3.7 – Structure of compounds more active for the breast cancer cell lines tested.

Previously, the work developed in the group had been focused on the evaluation of a small family of spirooxadiazoline oxindoles **26** in HCT116 and SW620 colon cancer cell lines. Some compounds of this small family were also tested in MCF-7 and MDA-MB-231 breast cancer cell lines (**Table 3.3**).

Table 3.3 – Comparison of cell viability of spirooxadiazoline oxindoles 26 in colon and breast cancer cell lines.

Cmpd	R_1	R_2	R_3	% cell viability at 50 µM			
				HCT116 *p53*$^{(+/+)}$	SW620	MCF-7	MDA-MB-231
26w	5-Br	H	H	>50	<50	>50	>50
26x	7-Br	H	H	>50	>50	>50	>50
26y	7-Cl	H	H	>50	>50	>50	>50
26z	6-Cl	H	*p*-OMe	>50	<50	>50	>50
26aa	5-Cl	*m*-Cl	*m*-Cl	>50	>50	>50	>50
26ab	5-Cl	H	*p*-Cl	>50	>50	>50	>50

IC_{50} was determined by MTT method after 48h. Each value is the mean (%±SD) of three experiments performed in triplicate.

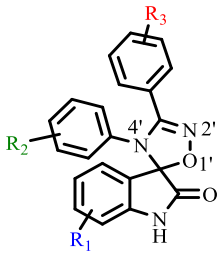
In general, the anti-proliferative activity of spirooxadiazoline oxindoles **26** is higher in SW620 than MCF-7 and MDA-MB-231 cell lines. SW620 is a colon cancer cell line with *mut* p53 with a different mutation of MDA-MB-231. SW620 has the mutation R273H, which in nucleotide 13798, a guanine is replaced by an adenine, so during the translation, a histidine is produced instead of an arginine. MDA-MB-231 has the mutation R280K, which in nucleotide 13819, a

guanine is also replaced by an adenine, so during the translation, a lysine is produced instead of a arginine.[18, 64]

For further optimizations, electron withdrawal groups should be tested. A compound with both *m*-chloro atom and *p*-methoxyphenyl group as R_3 and R_2, respectively, should be synthetized.

3.2.2. Comparison of spirooxadiazoline oxindole family with [1,2,4] and [1,3,4]-oxadiazole scaffolds

Previously, a family of spirooxadiazoline oxindoles with [1,2,4]-oxadiazole ring **25** was reported by our group.[13] To better understand the consequences of the inversion of the oxadiazole ring in families **25** and **26**, we evaluated the anti-proliferative activity in MCF-7 and MDA-MB-231 breast cancer cell lines (**Table 3.4**).



25

Table 3.4 – *In vitro* anti-proliferative activity of spirooxadiazoline oxindoles with [1,2,4]-oxadiazole core.

Cmpd	R_1	R_2	R_3	MCF-7 IC_{50} /µM	MDA-MB-231 IC_{50} /µM
25c	5-Br	3-Cl	H	18±8	>100
25d	5-Cl	4-Cl	4-Cl	34±9	24±10
25e	H	3-Cl	H	>100	>100
25f	5-Cl	4-Cl	3-Cl	34±8	43±4
25g	6-Cl	4-Cl	H	36±8	53±9
25h	H	4-F	H	>100	>100
25i	H	4-Cl	H	69±3	>100
25j	H	4-Cl	3-Cl	51±8	>100
25k	5-Cl	3-Cl	H	7±2	26±1
25l	5-Br	4-Cl	H	34±9	54±11
25m	5-Cl	4-Cl	H	44±2	58±1

IC_{50} was determined by MTT method after 48h. Each value is the mean (%±SD) of three experiments performed in triplicate.

Compound **25k** is the most potent spirooxadiazoline oxindole derivative against MCF-7 cell line. Spirooxadiazoline oxindoles **25** containing [1,2,4]-oxadiazole ring are, in general, more potent than the equivalent regioisomers **26** (**Fig. 3.9**).

Spirooxadiazoline oxindoles **25** were, previously, tested in HCT116 and SW620. The most potent compound was **25c** with an IC_{50} value of 4.7 µM for HCT116 *p53*$^{(+/+)}$ cell line. The corresponding spirooxadiazoline oxindole **26c** showed an IC_{50} value of 18 µM, which is 4 times less potent than HCT116 *p53*$^{(+/+)}$ cell line. The family of spirooxadiazoline oxindoles **25** has higher anti-proliferative activity in HCT116 and SW620 cell lines than MCF-7 and MDA-MB-231 cell lines.

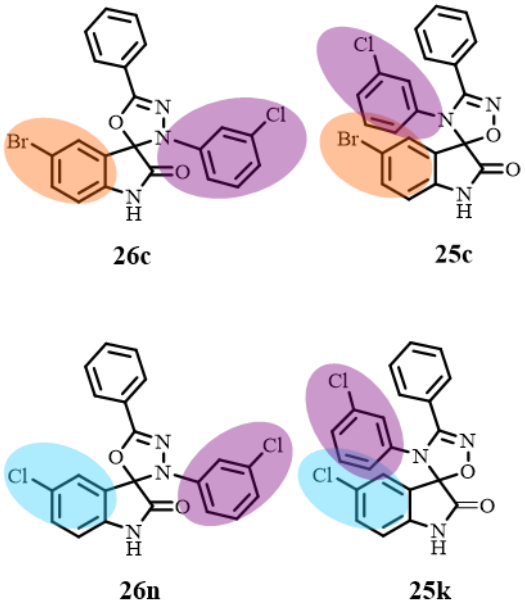


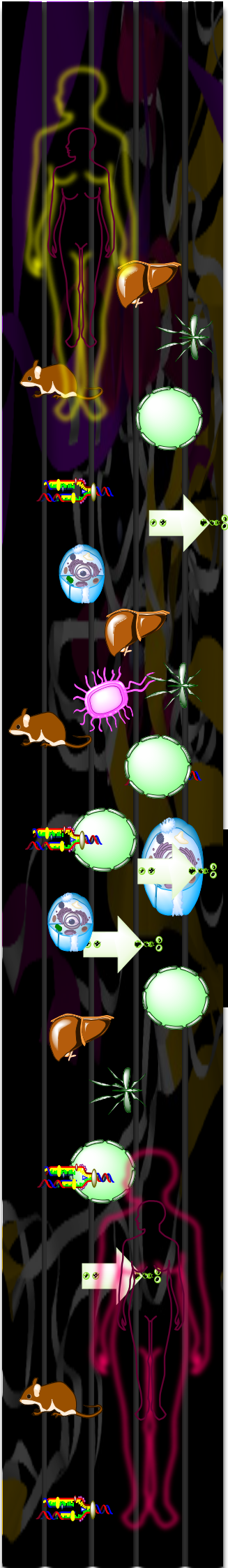
Fig. 3.8 – Most potent analogues from both spirooxadiazoline oxindole families 25 and 26.

3.3. Final remarks

The twenty spirooxadiazoline oxindoles **26** synthetized were tested in human breast adenocarcinoma cell lines. Compounds **26n** and **26u** showed an inhibition of 50% of cell growth in both MCF-7 and MDA-MB-231 cell lines. Furthermore, compounds **26c** and **26v** showed around 40% of inhibition of cell growth in just MDA-MB-231 cell line, which has p53 mutated. Compound **26h** showed to be more selective to MCF-7 cell line, showing 50% of cell viability. This family also shows to be more potent for SW620 colon cell lines.

Comparing spirooxadiazoline oxindoles **25** and **26**, compounds **25** showed to be more potent in the breast cancer cell lines tested.

Further chemical optimizations can be performed in spirooxadiazoline oxindoles **26** and the new derivatives should be tested in other cancer cell lines such as colon, lung, liver and ovaries.



Chapter 4

SMALL MOLECULES FOR TARGETING *P. FALCIPARUM*

Chapter 4. Small molecules for targeting *P. falciparum*

4.1. Introduction

Malaria is a hematoprotozoan parasitic infectious disease that usually affects African and Asian continents. This infection is transmitted by the bite of the female *Anopheles* mosquito. Despite all efforts, this disease is still one of the major causes of mortality and morbidity, especial in children and pregnant women. Half a century ago, there was a so-called eradication era when malaria was eliminated or almost entirely supressed in some parts of the world. However, in some of these areas, malaria appeared again. This phenomenon can be explained by a first-line antimalarial drug resistance. For this motif, it is urgent to develop new antimalarial drugs. Several synthetic and natural products have been discovered but their side effects or drug resistance have been making their development very difficult. For this reason, there is not an effective vaccine available and it is not expected for some more years.[65, 66]

Parasitic protozoan belonging to genus *Plasmodium* causes malaria. There are five species of the parasite: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium kowlesi*. The first two are the responsible for about 95% of the cases worldwide. The first one acts mostly in Africa, sub-Sahara Africa and East Asia, whereas the second acts in Indian subcontinent (**Fig. 4.1**).

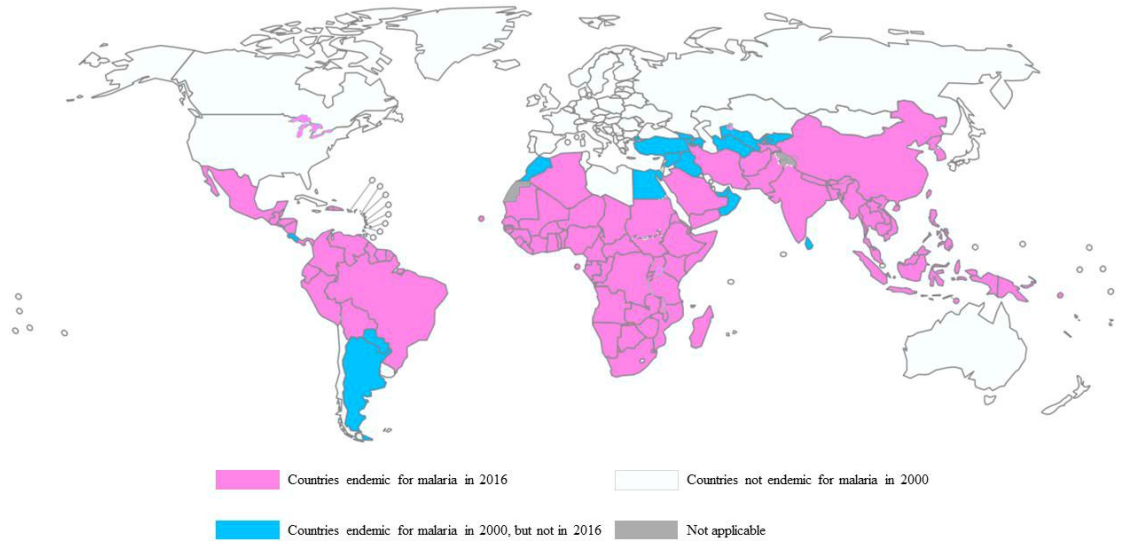


Fig. 4.1 – Countries endemic for malaria in 2000 and 2016. Since 2000, 17 countries are no longer endemic, mostly because of malaria control interventions[67]

According to WHO, in 2015, 97 countries had ongoing malaria transmission, having about 3.3 billion people in risk. It is estimated to have 149-303 million cases of malaria worldwide. 88%

are from Africa, 10% are in East Asia and 2% in Eastern Mediterranean region. It is estimated between 236000-635000 deaths, which 90% is in Africa and the rest of the 10% in the areas already identified. 65% of these deaths are children under five.[67]

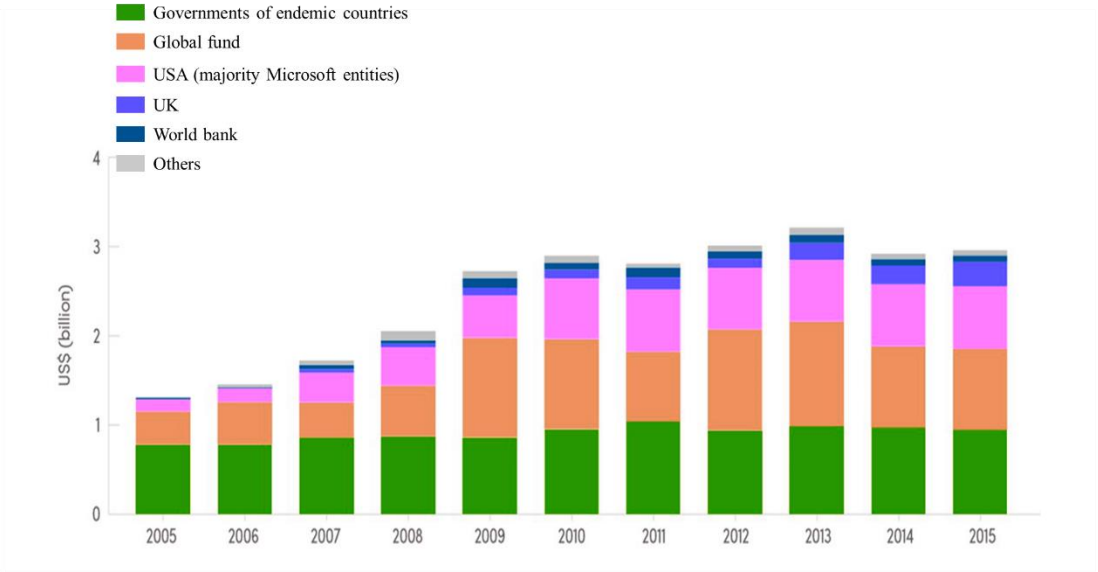


Fig. 4.2 – Increasing of investments. Governments of endemic countries provided 32% of total malaria funding in 2015, followed by international funds. USA and UK are in the ranking of the largest malaria funders, 35% and 16%, respectively.[67]

In most malaria-affected countries, people have less than 10€ per capita annually to spend in health care, so treatments that cost more than 0.50€ are unbearable.[66]

4.1.1. Life cycle of malaria parasite

Life cycle of malaria parasite involves two host viz. human and mosquito and it has two stages. The first one, called pre-erythrocity stage includes the bite of the *Plasmodium*-infected female *Anopheles* mosquito. Usually less than 10 sporozoites are inoculated into the human host by an infected mosquito.[66, 68] Sporozoites enter into the liver cells and grow into tissue schizonts which, after rupture, can release merozoites to the blood stream to begin the 48-hour asexual reproduction cycle. *P. vivax* and *P. ovale* can remain as hypnozoites (dormant condition) in the liver. After their release, merozoites multiply asexually in the erythrocytes and develop into immature ring stage trophozoites – asexual erythrocytic stage. In this stage the person shows clinical illness and complications of malaria. The mature schizonts burst and release merozoites into blood stream. Some of them discriminate into sexual erythrocytic stages and develop into immature gametocytes – precursors of male and female gametes. These are ingested by an *Anopheles* mosquito and start multiplication in the mosquito. They develop into sporozoites that are in mosquito's salivary glands.

All the symptoms can be associated with the asexual erythrocytic or blood stage parasites. These symptoms include diarrhoea, fever, headache, weakness, vomiting, cough and abdominal pain.[69] In *P. falciparum* malaria, if the drugs are readily available, the mortality is about 0.1%. If the parasite multiplicates without any interference, more severe symptoms appear as vital-organ dysfunction, acidosis and anaemia. At this stage, the mortality rises to about 20%.[66]

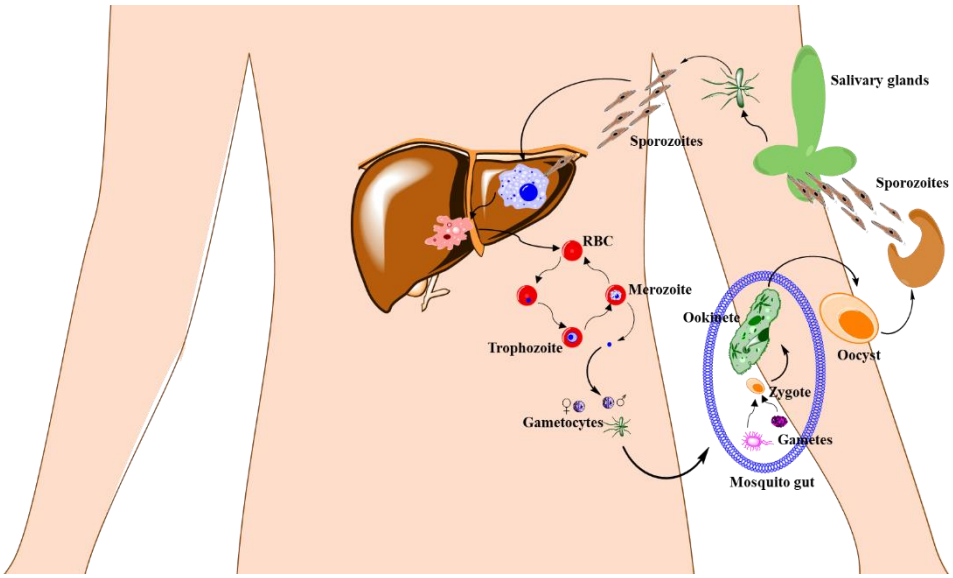


Fig. 4.3 – Life cycle of malaria parasite[adapted from [66]]

4.1.2. Strategies for antimalarial drug development

Most antimalarial drugs target the erythrocytic stage and not the hepatic stage, since it does not show any symptoms. Once the actual antimalarial drugs are ineffective, due to the *P. falciparum* resistance, the new strategies pass through develop drugs against all stages. The doses depend on the geographic location of the infection, the plasmodium species and also the severity of disease presentation. Ideally, a new antimalarial should have activity against the blood stages, be active against all drug-resistant parasites, not have cytotoxicity or genotoxicity and be efficient with a once-daily oral administration, have a novel mechanism of action and be cheap.[70, 71]

As explained before, the current antimalarial drugs have presented several problems as resistance, toxicity and high cost. New strategies have to be rethink, for example, replacement of chloroquine with a new drug or chloroquine with modifications to overcome the resistance problem associated (**Fig. 4.4**).[72, 73]

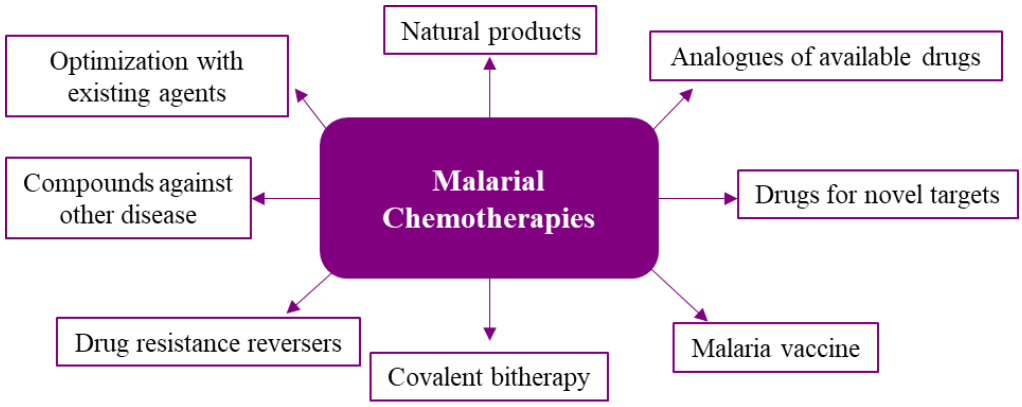
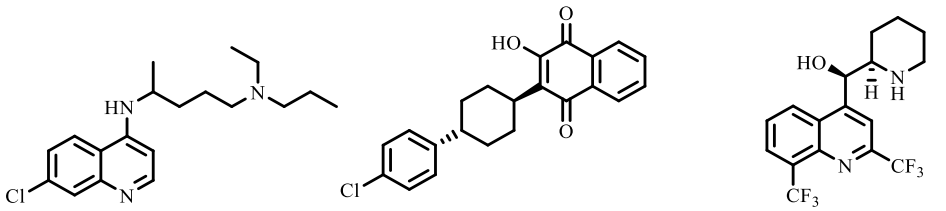


Fig. 4.4 – Several strategies for new antimalarial drugs.[73]

Some current strategies pass through combination therapies such as non-artemisinin-based and arteminin-basead combination therapy. In this strategy, chloroquine (**57**), atovaquone (**58**), mefloquine (**59**) and quinine (**5**) are combined with other drugs and antibiotics to overcome the resistance developed by *P. falciparum* for these drugs.[66, 74]



57, Chloroquine **58**, Atovaquone **59**, Mefloquine

Fig. 4.5 – Structural representation of chloroquine (57), atovaquone (58) and mefloquine (59).[73]

Another strategy usually used is the optimization of existing compounds. One example is the discovery of chloroquine (**57**) and mefloquine (**59**) that are all structural optimizations of the quinine.[75]

Covalent biotherapy is another strategy that consists in a covalent linking of different chemical moieties that different parts can have or the same target with different action mechanisms – hybrid molecules.[76] Trioxaquine is a drug from this class that has a 1,2,4-trioxane and a 4-aminoquinoline linked covalently with two different mechanisms: alkylation of heme with the trioxane moiety and binding of heme with the aminoquinoline moiety to inhibit hemozoin formation.[77-79] SAR-116242 is now in preclinical assessment by Safoni-Aventis and presents activity in sexual and asexual stages, showing activity against resistant strains of *P. falciparum*.

Several drugs used for the treatment of other diseases have also been used as antimalarial drugs. This is a very common strategy, since, with this approach, years of development are reduced, so their cost, pharmacokinetic profile and safety have already been tested. An example is atovaquone (**58**) was first used for prevention of pneumonia. However, used in combination with proguanil, it has a high activity against malaria.[80, 81]

Drug resistance is developed when a minimum amount of drug responsible for activity is not binding to the target. Chloroquine (**57**) is a first-line antimalarial therapy drug but resistance has decrease its efficacy. It was combined with drug resistance reversers which maintain the efficacy of chloroquine. These drug resistance reversers can be calcium channel blockers, such as verapamil and derivatives and diltiazem, can be tricyclic antidepressants, such as imipramine and desipramine, can be antihistamines and phenothiazines.

4.1.2.1. Natural products and derivatives

For centuries, natural products have been used as molecular templates for the development of new drugs, being used as lead compounds such as quinine (**5**), artemisinin (**1**) and spiroindolinones **21** (**Fig. 4.6**).[82]

Quinine (**5**) was the first chemical compound that occurred naturally in the bark of the cinchona tree for *P. falciparum* malaria. It was substituted for chloroquine, since it had several side effects. After a lot of use, the parasites developed resistance to quinine, so the monotherapy with it was abandoned. Since this moment, in the 1980's, combination therapy became the second line of treatment.

Artemisinin (**1**), a sesquiterpene lactone with a 1,2,4-trioxane ring was isolated from *Artemisia annua*. Because of its endoperoxide moiety, it forms free radicals when exposed to free iron. However, its limitations such as low solubility, bioavailability, short half-life and neurotoxicity, do not allow it to be use as monotherapy. Several optimizations and approaches have been made, developing new classes of antimalarial as trioxolane and tetraoxanes.[83, 84] Ozonide (OZ227) has a 1,2,4-trioxolane ring system and several combinations with other pharmacophores are currently in clinical trials. For example, azonide-piperaquine phosphate combination therapy is in Phase III and OZ439 has just completed Phase I and is currently entering Phase IIa.[85, 86]

4.1.2.1.1. Spiroindolinones

Spiroindolinones are an emerging class of antimalarial drugs, which possess excellent oral bioavailability and exceptional potency.

From a library of around 12000 natural products and derivatives, 250 compounds were selected with submicromolar activity against *P. falciparum.* From those, 17 compounds showed activity against multidrug-resistant forms of the parasite. From these compounds, compound **59** was chosen as initial compound for further optimizations.[70]

Compound **60** was a racemic mixture with moderate activity against *wt* and K1 strains of *P. falciparum* (100 mg/kg dose led to 96% decrease in parasitemia in the *P. berghei*-infected mouse). SAR studies showed that the 1R, 3S stereoisomer **61** was 250-fold more potent than the 1S, 3R stereoisomer against *P. falciparum*. The scaffold of the most potent stereoisomer was crucial for

the activity against this *Plasmodium*. The replacement of the bromine atom by a chlorine atom led to an increase in potency, pharmacokinetics and synthetic accessibility. The removal of a carbon of the azepine ring leading to a 6-membered ring increased the potency, leading to compound **62**.[70, 71]

The spiroindolinones were found to have good *in vitro* solubility and permeability, having no cytotoxicity or genotoxicity and do not bind to human receptor, kinases or ion channels. For these reasons, spiroindolinones are good candidates for new antimalarial drugs. [87]

For metabolic reasons, the benzene ring of the oxindole ring was modified. To avoid oxidation, halides were added to the oxindole ring. The addition of fluorine atom at position 7 improved the half-life of the compound **62** in the presence of liver microsomes.[70, 88]

The optimizations of *in vitro* studies led to a higher antimalarial activity in the *P. berghei*-infected mouse model than chloroquine and artesunate, prolonging the survival of the mice when a single administration dose of 30 mg/Kg.[89]

NITD609 (**21**) was showed to have IC_{50} values of 0.5-1.4 nM against *wt* and multidrug-resistant strains. Compound **21** was also showed to be effective for both *P. falciparum* and *P. vivax* with IC_{50} values less than 10 nM. The *in vitro* assays of *P. falciparum*, NITD609 was more active against schizonts. Besides being active in this phase, it also acts in gametocytes, which are responsible for the transmission of the parasite. Further studies showed that NITD609 decrease the oocyte count when added to the blood meal of mosquitos and it also is involved in sexual stages of transmission. However, compound **21** do not act against the liver stages of malaria.[90] With further studies, it was also demonstrated that compound **21** blocked the protein synthesis in *P. falciparum* within one hour, having a different mechanism action from other known drugs.[71]

NITD609 (**21**) is currently in Phase II clinical trials, acting in the blood forms of parasite and killing *P. falciparum* and *P. vivax* in nanomolar concentrations.[91]

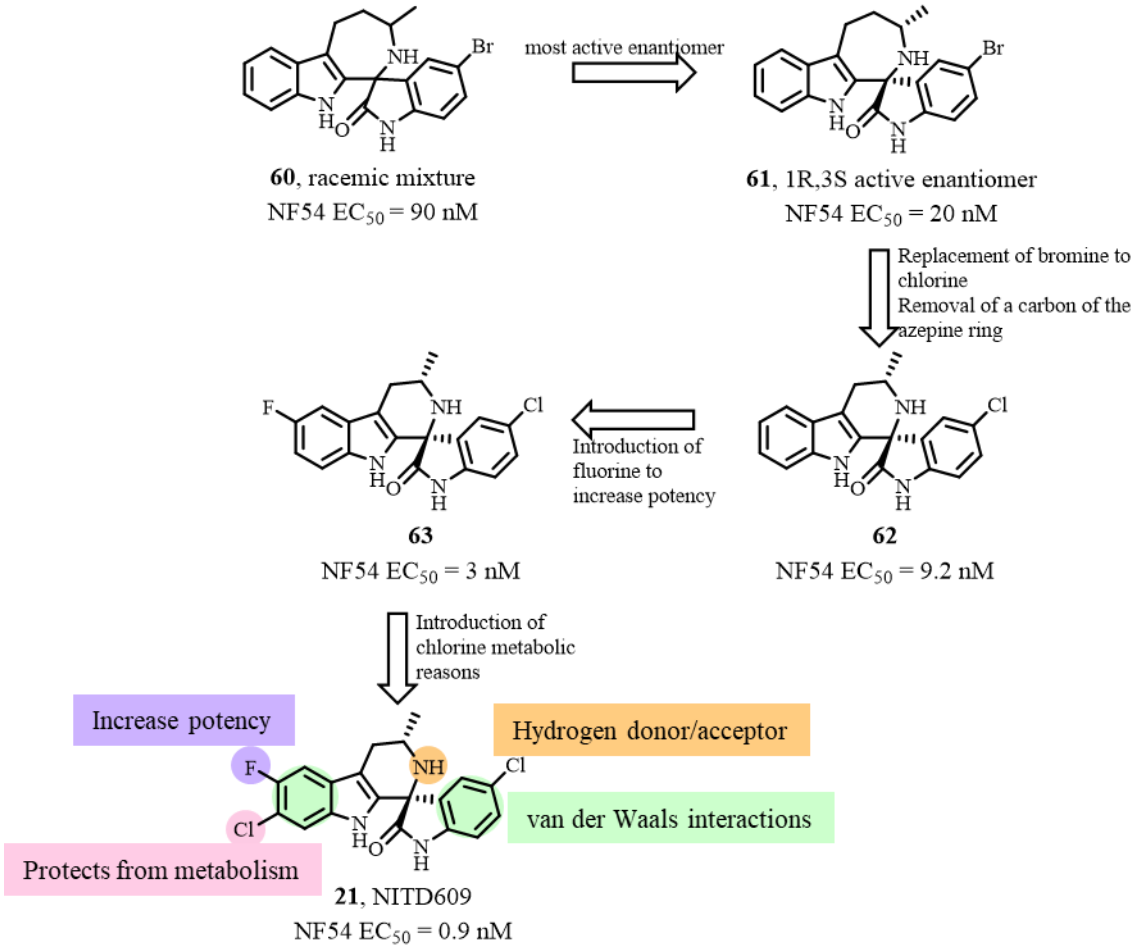


Fig. 4.6 – Optimization of spiroindolinones to NITD609 (21). [Adapted from [70]]

4.2. Screening against *Plasmodium falciparum* strains and SAR study

Several drugs for cancer treatment have been reported for the treatment of malaria, such as artemisinin and 7-chloroquinolinyl thioureas.[92, 93] For this reason, we decided to test the spirooxadiazoline oxindoles **26c-v** against chloroquine-resistant (W2) and chloroquine-sensitive (3D7) strains of *Plasmodium falciparum* (**Table 4.1**). The biological assays were performed by the Dr. Philip Rosenthal group in USA.

Table 4.1 – *In vitro* activity of spirooxadiazoline oxindole derivatives 26c-v against *Plasmodium falciparum*.

Cmpd	R_1	R_2	R_3	W2 IC_{50}/µM	3D7 IC_{50}/µM
26c	5-Br	Ph	*m*-Cl	6.71±0.86	>10
26d	5-Br	*m*-ClPh	H	>10	ND
26e	5-Cl	*m*-ClPh	H	>10	ND
26f	5-Br	*p*-ClPh	H	7.58±0.10	>10
26g	7-Cl	*m*-ClPh	H	>10	ND
26h	5-Br	*p*-OMePh	H	8.31±0.70	>10
26i	H	Ph	*p*-Cl	>10	ND
26j	7-Cl	*m*-ClPh	*m*-Cl	>10	ND
26k	H	*m*-ClPh	H	>10	ND
26l	7-Cl	Ph	*p*-Cl	>10	ND
26m	5-Cl	*p*-ClPh	H	>10	ND
26n	5-Cl	Ph	*m*-Cl	6.37±0.39	>10
26o	7-Cl	Ph	*m*-Cl	>10	ND
26p	7-Cl	*p*-ClPh	H	>10	ND
26q	H	Ph	*m*-Cl	>10	ND
26r	5-Cl	*p*-OMePh	H	>10	ND
26s	7-Cl	*p*-OMePh	H	>10	ND
26t	H	*p*-OMePh	H	>10	ND
26u	5-Br	t-Bu	H	4.07±0.36	8.44±0.83
26v	6-Cl	t-Bu	H	1.47±0.17	2.90±0.19

ND: Not determined

Six compounds showed activity against chloroquine-resistant strain and two compounds were potent against chloroquine-sensitive strain (**Fig. 4.7**).

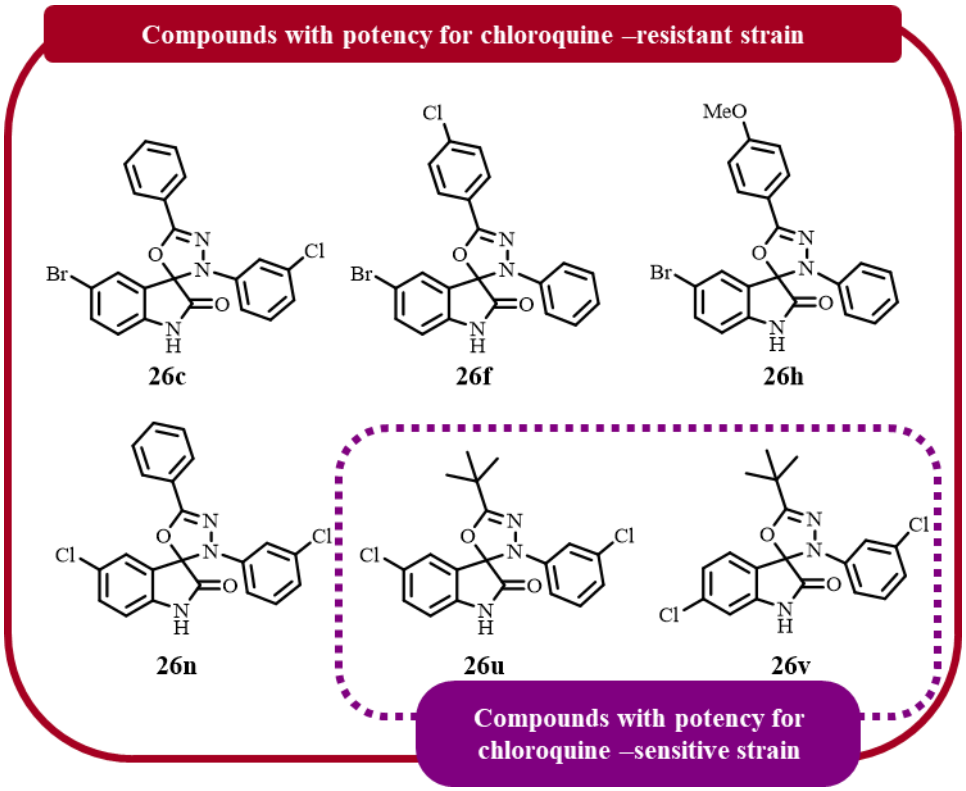


Fig. 4.7 – Compounds with potency against *Plasmodium falciparum*.

As observed in the anti-proliferative assays against breast cancer cell lines, compounds **26c**, **26f**, **26h**, **26n**, **26u** and **26v** showed activity against chloroquine-resistant strain of *Plasmodium falciparum*. Compound **26f** showed activity against this strain but did not show anti-proliferative activity against breast cancer cell lines. All these compounds have chlorine and bromine atoms at the position 5 or 6 of the oxindole ring. Compounds **26c**, **26h**, **26n**, **26u** and **26v** also have a *m*-chlorophenyl group in position 3'. Compounds **26f** and **26h** have a *para* substituted phenyl ring of the position 5'. Comparing **26f** and **26h** with **26m** and **26r**, when the bromine atom is replaced by a chlorine, the compound loses its activity. However, comparing this result with **26c** and **26n**, which have the same difference, the potency is maintained.

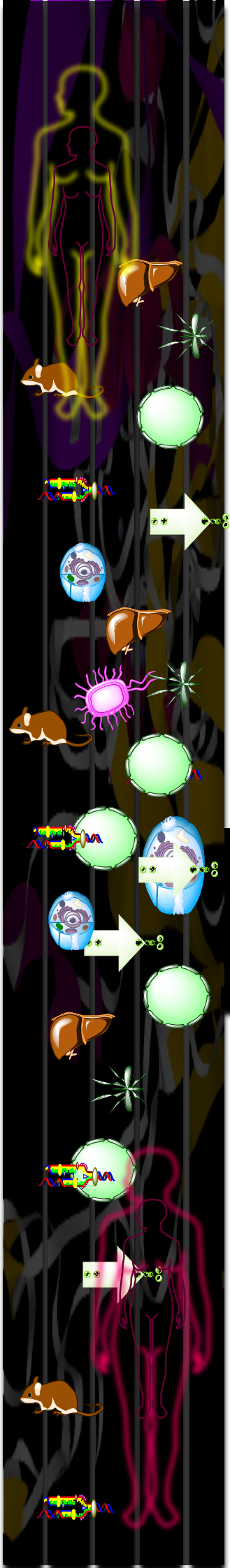
Compounds **26u** and **26v** are also active for chloroquine-sensitive strain, 3D7. These compounds have a *t*-butyl group in the position 5', instead of a phenyl ring. Considering this result, the alkyl group in that position seems to be crucial for the activity against the sensitive strain.

Variations of size and nature of the substituents in the position of 3' and 5' of the [1,3,4]-oxadiazole ring should be studied in further optimizations, for example, introduction of amine groups, more bulky alkyl groups and introduction of fluorine atoms. The amine group of the oxindole group can also be substituted to increase potency. Docking screenings to find possible targets with the obtained results would also be important to understand which optimizations could be made to increase the interactions with the target.

4.3. Final remarks

Since several anticancer drugs have been tested for the treatment of malaria, spirooxadiazoline oxindoles **26a-v** were tested in *P. falciparum* W2 and 3D7 strains. Compounds **26c**, **26f**, **26h**, **26n** and **26u-v** showed high potency against W2 strain. Moreover, **26u-v** showed high potency for 3D7 strain.

For future studies, spirooxadiazoline oxindoles **26** should also be tested against multidrug-resistant strain and in other malaria stages.



Chapter 5

FINAL CONSIDERATIONS

Chapter 5. Final considerations

Reactivation of p53 function in cancer cells is one of the most valuable and popular anticancer strategies.

In this thesis, a new library of spirooxadiazoline oxindoles containing a [1,3,4]-oxadiazole ring was developed. The synthetic route has three main steps: 1) reaction of substituted benzaldehydes with substituted phenylhydrazines to form hydrazones; 2) the chlorination of these hydrazones to obtain the corresponding hydrazonyl chlorides; 3) the reaction of the corresponding hydrazonyl chlorides with isatin derivatives to afford spirooxadiazoline oxindoles **26**.

According to the NMR spectra, this reaction is regioespecific. Just the [1,3,4]-oxindole regioisomer is obtained (confirmed by TLC and NMR) in most of the reactions with yields between 42 and 90%. [17, 24, 29]

The anti-proliferative activity of spirooxadiazoline oxindoles **26c-v** was evaluated in two breast cancer cell lines, MCF-7 and MDA-MB-231. Four compounds (**26c**, **26n**, **26u** and **26v**) showed around 50% of inhibition of cell growth. These spirooxadiazoline oxindoles have bromine or chlorine in positions 5 and 6 of the oxindole ring and the position 3' of the oxadiazole ring substituted by a *m*-chlorophenyl group. These three positions showed to be important for activity. Compound **26h** showed selectivity against MCF-7 cell line over MDA-MB-231 cell line. This compound has a *para* substituted phenyl ring in position 5' of the oxadiazole ring. Some derivatives of spirooxadiazoline oxindoles **26** have been previously evaluated in HCT116 and SW620 cell lines, showing good activity for SW620 cell line. Here, it was shown that spirooxadiazoline oxindoles **26** lose their activity in MCF-7 and MDA-MB-231 cell lines. So, this family can be specific for SW620 cell line, which has a R280K mutation in p53.[18]

Furthermore, this family was compared with the spirooxadiazole oxindole family **25** previously reported by our group.[16] Spirooxadiazoline oxindoles **25** showed higher anti-proliferative activity against the two breast cancer cell lines used in the assays. Compound **25k** was the most potent. Comparing with previous results, spirooxadiazoline oxindoles **26** and **25** have higher anti-proliferative activity in colon cancer cell lines (HCT116 and SW620) than in breast cancer cell lines (MCF-7 and MDA-MB-231).

It has been reported several compounds showing dual activity against cancer and malaria. For this reason, spirooxadiazoline oxindoles **26** were also tested against *Plasmodium falciparum*, W2 and 3D7 strains. Six compounds showed activity lower than 9 µM for W2 strain and two compounds showed activity against 3D7 strain. All these compounds have the same substituents of the ones for cancer (*m*-chlorophenyl and *p*-chlorophenyl rings, *t*-butyl group and halogens at position 5 and 6 of the oxindole core), which supports that cancer and malaria have therapeutically targets in common. Comparing activities, the *m*-chlorophenyl ring in position 3' is crucial for

binding as well as the substitution of position 5 in the oxindole ring. Compounds without these features were not active. The *para* substitution of phenyl ring of position 5' and alkyl groups showed to be important for potency in W2 strain, which means bulk groups should be used to fill the binding site. On the other hand, for 3D7 strain, compounds with aromatic rings in this position lost their activity, so the *t*-butyl group seems to be crucial for this strain of *Plasmodium falciparum*.

Six promising compounds have been achieved with this thesis. Further optimizations should be done to increase the potency of this family for both cancer and malaria (**Fig. 5.1**). Asymmetric synthesis can be performed for the most active compounds **26u** and **26v**.

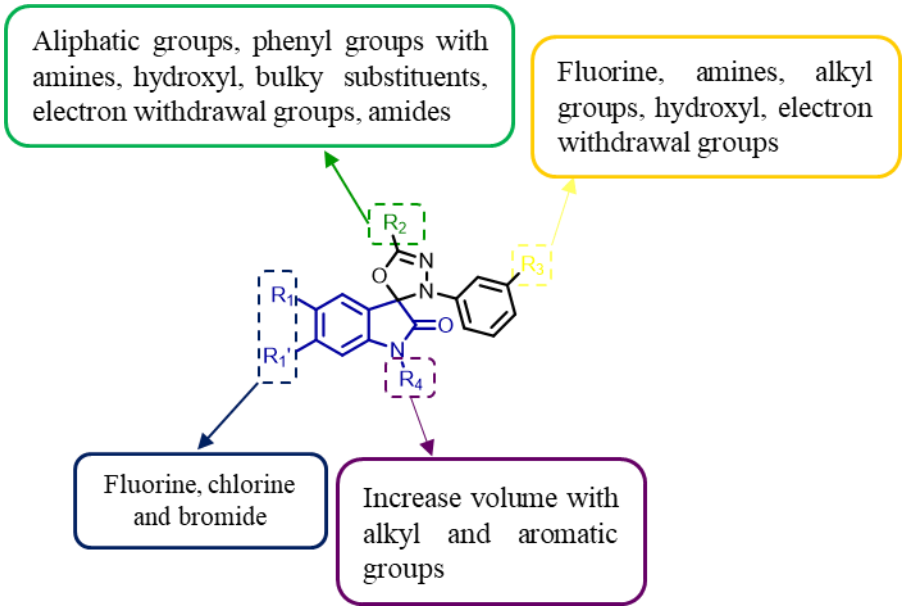
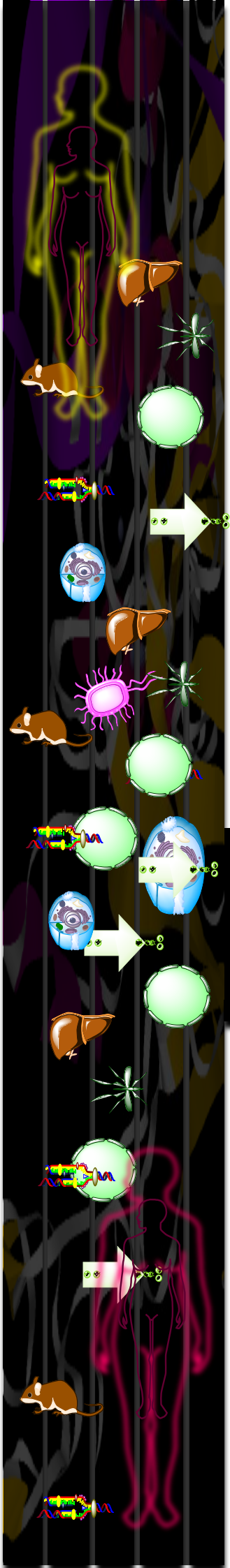


Fig. 5.1 – Further optimizations that can be performed to increase potency against cancer and malaria.

Spirooxadiazoline oxindoles **26** can be also tested in other cancer cell lines, like colon, stomach, lung and ovaries.

Further assays can be also performed in malaria, such as, against the multidrug-resistant strain K1 of *P. falciparum*. Other stages of malaria should also be tested, such as liver stage. In addition, the cytotoxicity of the spirooxadiazoline oxindoles **26** in non-cancer cell lines should be evaluated.



Chapter 6

EXPERIMENTAL SECTION

Chapter 6. Experimental Section

6.1. Chemistry: solvents and instruments

All chemical and solvents were obtained from commercial suppliers and were normally used without further purification. CH_2Cl_2 was dried over CaH_2 and distilled, TEA was dried over KOH, distilled and stored with it, EtOAc was dried over $CaCO_3$ and distilled.

Thin layer chromatography was performed using Merck Silica Gel 60 F254 aluminium plates and visualized by UV light. Flash column chromatography was performed on Merck Silica Gel (200-400 mesh ASTM). Preparative TLC was performed on Merck Silica Gel 60 GF254 over glass plates with 0.5 and 1 mm thickness. All reactions were performed under N_2 atmosphere.

^1H and ^{13}C NMR spectra were recorded on a Bruker Fourier 300 at 300 and 75 MHz, respectively. ^1H and ^{13}C chemical shifts (δ) are expressed in parts per million (ppm) using the solvent as internal reference, and proton coupling constants (J) in hertz (Hz). ^1H spectral data are reported as follows: chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; dt, doublet of triplets, and br, broadened), coupling constant, and integration.

The infrared spectra were collected on a Shimadzu FTIR Affinity-1 spectrophotometer. The spectra were determined using thin films in a NaCl pellet. Only the most significant absorption bands are reported.

The LC-MS data were collected on performed in a Waters Alliance 2695 HPLC with a LiCrospher® 100 RP-18 column (125 x 4 mm; 5 µm) at 35 ºC, using as mobile phase ACN:H_2O (70:30), and employing a photodiode array detector to scan wavelength absorption from 230 to 650 nm; MS experiments were performed on Micromass® Quattro Micro triple quadrupole (Waters®, Ireland) with an electrospray in positive ion mode (ESI+), ion source at 120 ºC, capillary voltage of 3.0 kV and source voltage of 30V.

Melting points were determined using a Kofler camera Bock monoscope M and are uncorrected.

Some hydrazonyl chlorides, such as **29h** and **29i**, have been previously prepared by Carlos Ribeiro and Dário Silva and were in stock in the freezer at -25ºC.[23]

The microwave-assisted reactions were performed using a Qlabo CEM Discovery equipment.

6.1.1. Procedure for the synthesis of 6-chloroindoline-2,3-dione (**27a**)

A mixture of 6-chlorooxindole (1.0 equiv) and $CuBr_2$ (4.6 equiv) in 63 mL of EtOAc was heated at reflux. After 6 h, the solvent was removed under reduced pressure and a mixture of MeOH:H_2O (4:1 v/v) was added. The mixture was heated at reflux for 3 h and, after removal of

the solvent, the residue was purified by flash chromatography to obtain an orange solid (652.1 mg, 42%). The NMR is in accordance with the one described in the literature [31].

6.1.2. General procedure for the synthesis of hydrazones **30a-g**

A mixture of phenylhydrazine derivative (1.0 equiv) and benzaldehyde derivative (1.2 equiv) in aqueous ethanol 20% (2 mL/mmol of phenylhydrazine) was stirred at room temperature in the dark for 2-3 hours. The precipitate formed was filtered and washed with cold aqueous ethanol 20% to afford hydrazones **30a-g** in yields between 55 and 98%. When the starting phenylhydrazine is in chloridrate form, triethylamine (1.0 equiv) was added 15 minutes before adding the corresponding benzaldehyde.[24] The NMR spectra are in accordance with the ones described in the literature (**30a** [25], **30b** [28], **30c**, **30e** [23], **30d** [26], **30f** [27], **30g** [17]).

6.1.3. General procedure for the synthesis of hydrazonyl chlorides **29a-g**

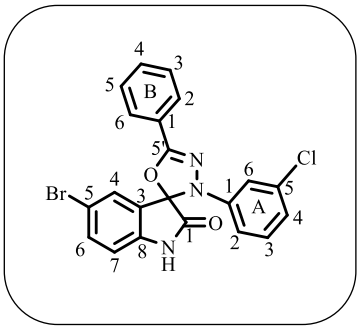
To NCS (3.0 equiv) in CH_2Cl_2 (3.5 mL/mmol of hydrazone) at 0 °C was added methyl sulfide (6.0 equiv) over 5 minutes. After stirring for 15 minutes, the reaction was further cooled to –78°C. Then the corresponding hydrazone (1.0 equiv) dissolved in CH_2Cl_2 (1 mL/mmol of hydrazone) was added. The reaction was stirred at –78°C for 1 h, and then slowly allowed to warm to room temperature over 3h. The reaction was quenched by addition of cold water. The organic layer was then washed with brine once, saturated sodium sulfite aqueous solution twice and water. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated to afford the corresponding hydrazonyl chlorides **29a-g** in yields between 49 and 92%.[29, 30] The NMR spectra are in accordance with the ones described in the literature (**29a**, **29b**, **29c**, **29d, 29f** [30], **29e** [23], **29g** [29]).

6.1.4. General procedure for the synthesis of spirooxadiazoline oxindoles **26c-v**

Hydrazonyl chloride (2.0 equiv) was dissolved in CH_2Cl_2 (10 mL/mmol of isatin) previously dried with CaH. The corresponding isatin (1.0 equiv) was added and the solution was stirred for 15 min. Then, TEA (2.0 equiv) was added dropwise. The reaction was stirred overnight, at room temperature and N_2 atmosphere. The reaction was quenched with cold water and the organic layer was washed with brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. All final compounds were purified by flash chromatography and/or preparative TLC and recrystallized with hot CH_2Cl_2 and room temperature *n*-heptane.

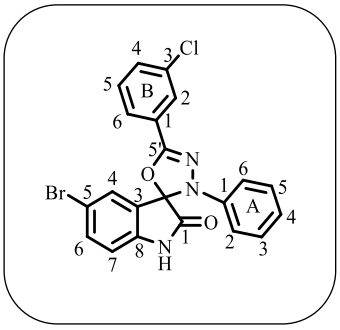
*Synthesis of 5-bromo-3'-(3-chlorophenyl)-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26c**)*

To a round bottom flask, was added 155.6 mg of 30a (0.6 mmol, 2.0 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 68.0 mg of 5-bromoisatin (0.3 mmol, 1.0 equiv) was added and after 15 min of stirring, 44.0 µL of TEA (0.3 mmol, 1.0 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 10 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The obtained oil was purified by preparative TLC, using as eluent *n*-hexane: ethyl acetate (7:3) to obtain, after recrystallization, a yellow solid (87.3 mg, 64%). **Mp:** 217-222ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.23 (br s, 1H, NH), 7.91 (dd, *J* = 7.6, 1.7 Hz, 2H, H-B_{arom}), 7.83 (d, *J* = 1.7 Hz, 1H, H_4), 7.70 (dd, *J* = 8.4, 1.9 Hz, 1H, H_{indole}), 7.52 (m, 3H, H-B_{arom}), 7.16 (m, *J* = 8.0, 3.9 Hz, 2H, H-A_3, H-A_5), 7.09 (m, 1H, H-A_{arom}), 6.87 (dd, *J* = 8.0, 1.1 Hz, 1H, H-A_{arom}), 6.63 (dd, *J* = 8.2, 1.5 Hz, 1H, H-A_{arom}). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm) 171.06 (C_1), 153.04 ($C_{5'}$), 144.26 (C_{A-1}), 142.85 ($C_{q\text{-}indole}$), 136.75 (CH_{indole}), 135.52 ($C_{q\text{-}indole}$), 132.11 ($CH_{B\text{-}arom}$), 131.66 (CH_{indole}), 130.14 (CH_{indole}), 129.81 ($CH_{B\text{-}arom}$), 127.28 ($CH_{B\text{-}arom}$), 126.29 (C_{A-5}), 125.39 (C_{B-1}), 121.44 ($CH_{A\text{-}arom}$), 116.45 (C_5), 114.79 ($CH_{A\text{-}arom}$), 114.58 ($CH_{A\text{-}arom}$), 112.45 ($CH_{A\text{-}arom}$), 95.42 (C_2). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3254 (NH), 1743 (C=O), 1618, 1593 (C=N), 1481, 1373, 1199, 1095, 1060,1024, 819. **ESI-MS m/z**: 456.1 $[M+H]^+$.



*Synthesis of 5-bromo-5'-(3-chlorophenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26d**)*

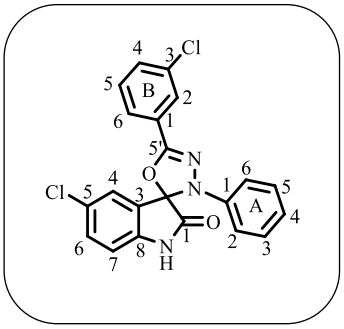
To a round bottom flask, was added 133.2 mg of 30f (0.5 mmol, 2.2 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 50.9 mg of 5-bromoisatin (0.2 mL, 1.0 equiv) was added and after 15 min of stirring, 49.0 µL of TEA (0.4 mmol, 1.6 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The obtained oil was purified by preparative TLC, using as eluent *n*-hexane: ethyl acetate (3:2) to afford, after recrystallization, a yellow solid (29.5 mg, 61%). **Mp:** 225-227ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.10 (br s, 1H, NH), 8.42 (m, 2H, H-A_{arom}), 7.77 (m, 1H, H_{indole}), 7.67 (dd, *J* = 8.3, 1.6 Hz, 1H, H_{indole}), 7.53 (dd, *J* = 7.75, 5.74 Hz, 2H, H-B_{arom}), 7.13



(m, 3H, H_4, H-A_{arom}), 6.86 (dd, *J* = 6.7, 7.9 Hz, 3H, H-A_{arom}). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm) 172.08 (C_1), 152.38 ($C_{5'}$), 143.67 ($C_{A\text{-}1}$), 137.28 (CH_{indole}), 135.99 ($C_{q\text{-}B1}$), 132.36 (CH_{indole}), 132.28 ($CH_{B\text{-}arom}$), 130.88 ($CH_{A\text{-}arom}$), 130.83 ($C_{q\text{-}B3}$), 128.47 ($C_{q\text{-}indole}$), 127.45 (CH_{Barom}), 127.38 (CH_{Barom}), 126.20 (CH_{Barom}), 122.94 ($CH_{A\text{-}arom}$), 117.02 ($C_{q\text{-}5}$), 115.77 ($CH_{A\text{-}arom}$), 115.15 (CH_{indole}), 96.72 (C_2). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3282 (NH), 1743 (C=O), 1658, 1600 (C=N), 1570, 1494, 1464, 1292, 1246, 1076, 918, 885. **ESI-MS m/z**: 456.1 $[M+H]^+$.

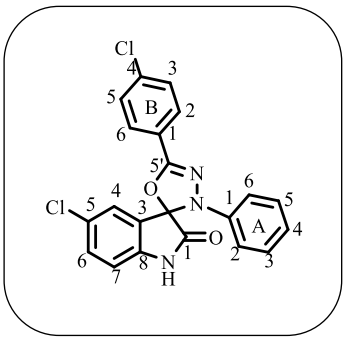
*Synthesis of 5-chloro-5'-(3-chlorophenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26e**)*

To a round bottom flask, was added 93.8 mg of 30f (0.4 mmol, 2.2 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 28.7 mg of 5-chloroisatin (0.2 mmol, 1.0 equiv) was added and after 15 min of stirring, 42.0 µL of TEA (0.3 mmol, 1.9 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The obtained oil was purified by flash chromatography using as eluent *n*-hexane: ethyl acetate (4:1) to obtain, after recrystallization, a yellow solid (55.2 mg, 85%). **Mp:** >300ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 9.80 (s, 1H, NH), 8.02 – 7.93 (m, 2H, 2H$_{arom}$), 7.66 – 7.52 (m, 3H, H_4, 2H$_{B\text{-}arom}$), 7.25 – 7.15 (m, 4H, H$_{A\text{-}2,6}$, H$_{B\text{-}arom}$), 7.00 – 6.94 (m, *J* = 8.6, 1.0 Hz, 2H, H$_{A\text{-}arom}$), 6.85 – 6.77 (m, 1H, H$_{A\text{-}arom}$).**^{13}C NMR** (75 MHz, acetone-d_6) **δ** 172.22 (C_1), 152.40 ($C_{5'}$), 143.69 ($C_{B\text{-}1}$), 143.21 ($CH_{B\text{-}arom}$), 136.00 ($C_{q\text{-}indole}$), 134.37 ($CH_{B\text{-}arom}$), 132.35 ($CH_{B\text{-}arom}$), 132.28 ($CH_{B\text{-}arom}$), 130.87 (CH_{indole}), 129.93 ($C_{q\text{-}indole}$), 128.47 ($C_{q\text{-}5}$), 128.03 (CH_{arom}), 127.38 (CH_{arom}), 127.11 ($C_{A\text{-}1}$), 126.20 (CH_{indole}), 122.95 ($CH_{A\text{-}arom}$), 115.82 ($CH_{A\text{-}arom}$), 114.71 ($CH_{A\text{-}arom}$), 96.82 (C_2). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3283 (NH), 1726 (C=O), 1658, 1602 (C=N), 1570, 1494, 1469, 1296, 1247, 1178, 1085, 920, 885. **ESI-MS m/z**: 410.2 $[M+H]^+$.



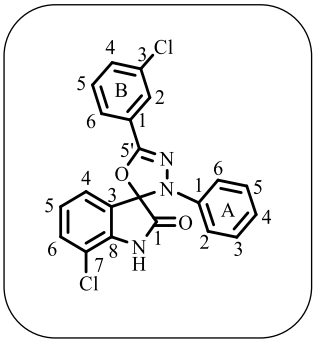
*Synthesis of 5-bromo-5'-(4-chlorophenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26f**)*

To a round bottom flask, was added 333.8 mg of 30b (1.3 mmol, 1.6 equiv) which was dissolved in 6 mL of dry CH_2Cl_2. Then, 180.1 mg of 5-bromoisatin (0.8 mmol, 1.0 equiv) was added and after 15 min of stirring, 168.0 µL of TEA (1.2 mmol, 1.5 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 10 mL of cold water and the organic layer was washed with 30 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. It was obtained yellowish brown oil that was purified by flash chromatography, using as eluent *n*-hexane: ethyl acetate (7.5:2.5) to afford, after recrystallization, a yellow solid (186.1 mg, 51%). **Mp:** 219-221ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.36 (br s, 1H, NH), 7.89 (m, 2H, H-B_{arom}), 7.51 (m, 5H, H-B_{arom}, H-A_{arom}, H_{arom}), 7.17 (m, 3H, H-A_{arom}, H_{arom}), 6.89 (m, 2H, H-A_{arom}). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm) 172.14 (C_1), 154.08 ($C_{5'}$), 142.87 ($C_{q\text{-indole}}$), 142.07 ($C_{A\text{-}1}$), 134.40 (CH_{indole}), 132.77 (CH_{indole}), 130.77($CH_{A\text{-}arom}$), 130.55 ($CH_{B\text{-}arom}$), 127.94 ($CH_{B\text{-}arom}$), 127.15 ($C_{B\text{-}4}$), 126.84 ($C_{B\text{-}1}$), 126.44 (CH_{indole}), 126.39 ($CH_{A\text{-}arom}$), 126.22 ($C_{q\text{-indole}}$), 117.99 ($C_{q\text{-}5}$), 117.27 ($CH_{A\text{-}arom}$), 97.03 (C_2). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3275 (NH), 1743 (C=O), 1620, 1597 (C=N), 1491, 1473, 1450, 1373, 1315, 1174, 1145, 1087, 1022, 823. **ESI-MS m/z**: 410.2 $[M+H]^+$.



*Synthesis of 7-chloro-5'-(3-chlorophenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26g**)*

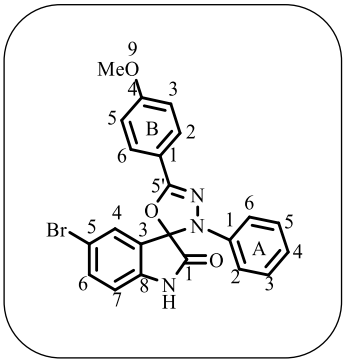
To a round bottom flask, was added 215.5 mg of 30f (0.8 mmol, 2.3 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 64.0 mg of 7-chloroisatin (0.4 mmol, 1.0 equiv) was added and after 15 min of stirring, 93.0 µL of TEA (0.7 mmol, 1.9 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. It was obtained an oil which was purified by flash chromatography, using as eluent *n*-hexane: ethyl acetate (3:2) to afford, after recrystallization, a yellow solid (77.0 mg, 54%). **Mp:** 215-218ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.41 (br s, 1H, NH), 7.83 (m, 2H, H_{indole}), 7.58 (m, 4H, H_{indole}, 3$H_{B\text{-}arom}$), 7.25 – 7.17 (m, 3H, $H_{B\text{-}arom}$, $H_{A\text{-}arom}$), 6.94 – 6.86 (m, 3H, $H_{A\text{-}arom}$). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm) 172.27 (C_1), 152.43 ($C_{5'}$), 143.70 ($C_{A\text{-}1}$), 142.04 ($C_{q\text{-indole}}$) 136.04 ($C_{q\text{-indole}}$), 134.33 (CH_{Barom}), 132.40 (CH_{Barom}),132.35 (CH_{indole}), 130.90 ($CH_{A\text{-}arom}$), 128.39 ($C_{q\text{-}7}$), 127.36 (CH_{indole}), 127.07 ($C_{B\text{-}1}$), 126.43 (CH_{Barom}), 126.38 (CH_{Barom}), 126.19 (CH_{indole}), 123.02



($CH_{A\text{-}arom}$), 117.89 ($C_{B\text{-}3}$), 115.89 ($CH_{A\text{-}arom}$), 97.34 (C_2). **IR** (NaCl) ν_{max} (cm^{-1}) 3263 (NH), 1736 (C=O), 1620, 1595 (C=N), 1496, 1473, 1363, 1261, 1220, 1188,1147, 1060,1020, 939, 882. **ESI-MS m/z**: 456.1 $[M+H]^+$.

*Synthesis of 5-bromo-5'-(4-methoxyphenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26h**)*

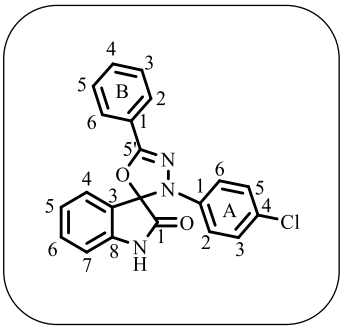
To a round bottom flask, was added 199.5 mg of 30e (0.8 mmol, 2.2 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 79.6 mg of 5-bromoisatin (0.4 mmol, 1.0 equiv) was added and after 15 min of stirring, 106.0 µL of TEA (0.8 mmol, 2.2 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. It was obtained an oil that was purified by preparative TLC, using as eluent a gradient *n*-hexane 100% to *n*-hexane: ethyl acetate from (3:2) to afford, after recrystallization, a yellow solid (51.7 mg, 62%). **Mp:** 164-166ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** 9.57 (s, 1H, NH), 8.02 – 7.96 (m, 2H, $H_{B\text{-}arom}$), 7.78 (dd, *J* = 8.4, 2.1 Hz, 1H, H_4), 7.70 – 7.67 (m, 1H, H_{indole}), 7.22 – 7.13 (m, 2H, $H_{B\text{-}arom}$), 7.08 – 7.02 (m, 3H, $H_{A\text{-}2,6,}$ H_{indole}), 6.97 – 6.92 (m, 2H, $H_{A\text{-}arom}$), 6.82 – 6.75 (m, 1H, $H_{A\text{-}arom}$), 3.89 (s, 3H, H_9). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** 184.52 (C_1), 167.82 ($C_{5'}$), 164.23 ($C_{q\text{-}Barom}$), 159.99 ($C_{q\text{-}Barom}$), 151.63 ($C_{q\text{-}indole}$), 151.20 ($C_{q\text{-}indole}$), 142.00 (CH_{indole}), 130.80 ($CH_{B\text{-}arom}$), 130.38 ($CH_{B\text{-}arom}$), 128.72 (CH_{indole}), 127.13 ($C_{A\text{-}1}$), 121.27 ($CH_{A\text{-}arom}$), 116.56 ($C_{q\text{-}indole}$), 115.96 (CH_{indole}), 115.34 ($CH_{A\text{-}2.6}$), 114.85 ($CH_{A\text{-}3,5}$), 96.72 (C_2), 56.61 (CH_9). **IR** (NaCl) ν_{max} (cm^{-1}) 3275 (NH), 1743 (C=O), 1608 (C=N), 1508, 1494, 1467, 1255, 1175, 1026, 835.



*Synthesis of 3'-(4-chlorophenyl)-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26i**)*

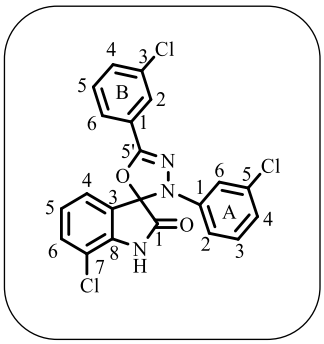
To a round bottom flask, was added 178.7 mg of 30h (0.7 mmol, 2.0 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 49.7 mg of isatin (0.3 mmol, 1.0 equiv) was added and after 15 min of stirring, 93.0 µL of TEA (0.7 mmol, 2.0 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The yellow oil was purified by flash chromatography, using as eluent gradient *n*-hexane to *n*-hexane: ethyl acetate (4:1) to afford, after recrystallization, a yellow solid (86.7 mg, 68%). **Mp:** 134-136ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.08 (br s, 1H, NH), 7.91 (m, 2H, H_{indole}), 7.52 (m, 5H; H_{indole}, $4H_{B\text{-}arom}$),

7.06 (m, 4H, H_{indole}, $3H_{A\text{-}arom}$), 6.84 (m, 1H, $H_{A\text{-}arom}$), 6.65 (m, 1H, $H_{A\text{-}arom}$). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm) 172.17 (C_1), 154.20 ($C_{5'}$), 145.22 ($C_{q\text{-}indole}$), 144.35 ($C_{q\text{-}B}$), 136.14 ($C_{q\text{-}indole}$), 134.66 (CH_{indole}), 132.75 (CH_{indole}), 132.23 ($CH_{A\text{-}arom}$), 130.52 ($CH_{B\text{-}arom}$), 127.93 ($CH_{B\text{-}arom}$), 127.86 (CH_{indole}), 126.27 ($C_{q\text{-}B}$), 125.31 (CH_{indole}), 124.82 ($C_{A\text{-}1}$), 121.87 ($CH_{A\text{-}arom}$), 115.38 ($CH_{A\text{-}arom}$), 113.24($CH_{A\text{-}arom}$), 113.21($CH_{A\text{-}arom}$), 96.57 (C_2). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3275 (NH), 1743 (C=O), 1656, 1600 (C=N), 1597, 1543, 1496, 1471, 1433, 1288, 1244, 1201, 1089, 1008, 929, 883.



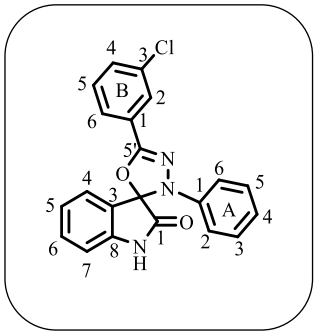
*Synthesis of 7-chloro-3',5'-bis(3-chlorophenyl)-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26j**)*

To a round bottom flask, was added 123.7 mg of 30e (0.4 mmol, 2.4 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 35.6 mg of 7-chloroisatin (0.3 mmol, 1.0 equiv) was added and after 15 min of stirring, 105.0 µL of TEA (0.8 mmol, 3.8 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The yellow oil was purified by flash chromatography, using as eluent gradient *n*-hexane to *n*-hexane: ethyl acetate (4:1) to afford, after recrystallization, a yellow solid (35.7 mg, 67%). **Mp:** 219-220ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.48 (s, 1H, NH), 7.88 (ddd, *J* = 8.7, 4.6, 1.9 Hz, 2H, $H_{B\text{-}arom}$), 7.65 – 7.50 (m, 3H, $3H_{indole}$), 7.26 – 7.16 (m, 2H, $H_{B\text{-}arom}$), 7.13 (t, *J* = 2.1 Hz, 1H, $H_{A\text{-}arom}$), 6.88 (dd, *J* = 16.5, 7.9 Hz, 2H, $H_{A\text{-}arom}$), 6.63 (dd, *J* = 8.3, 2.2 Hz, 1H, $H_{A\text{-}arom}$). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm) 171.80 (C_1), 153.04 ($C_{5'}$), 144.75 ($C_{q\text{-}Barom}$), 136.31 ($C_{q\text{-}indole}$), 134.63 ($C_{q\text{-}indole}$), 134.19 ($C_{q\text{-}7}$), 132.70 (CH_{indole}), 132.47 ($CH_{B\text{-}arom}$), 130.84 ($C_{q\text{-}B3}$), 130.51 (CH_{indole}), 127.79 ($CH_{B\text{-}arom}$), 127.55 ($CH_{B\text{-}arom}$), 126.54 ($CH_{B\text{-}arom}$), 126.41 (CH_{indole}), 122.68 ($C_{q\text{-}A1}$), 122.55 ($CH_{A\text{-}arom}$), 118.01 ($C_{q\text{-}A5}$), 115.81 ($CH_{A\text{-}arom}$). 115.68 ($CH_{A\text{-}arom}$), 113.37 ($CH_{A\text{-}arom}$), 97.05 (C_2). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3225 (NH), 1747 (C=O), 1595 (C=N), 1593, 1570, 1475, 1450, 1375, 1303, 1263, 1186, 1145, 1101, 1058, 1022, 960, 864. **ESI-MS m/z**: 444.1 $[M+H]^+$.



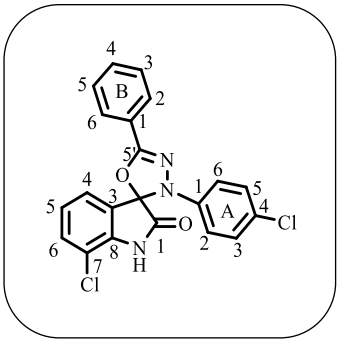
Synthesis of 5'-(3-chlorophenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one ***(26k)***

To a round bottom flask, was added 162.3 mg of 30f (0.6 mmol, 1.6 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 56.6 mg of isatin (0.4 mmol, 1.0 equiv) was added and after 15 min of stirring, 75.0 µL of TEA (0.7 mmol, 1.4 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The yellow oil was purified by flash chromatography, using as eluent gradient *n*-hexane to *n*-hexane: ethyl acetate (7:3) and *p*-TLC, using as eluent *n*-hexane: ethyl acetate (3:2) to afford, after recrystallization, a yellow solid (115.4 mg, 80%). **Mp:** 216-219ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.0 (br s, 1H, NH), 8.02 – 7.80 (m, 2H, $2H_{indole}$), 7.62 – 7.48 (m, 4H, $2H_{indole}$, $2H_{A\text{-}arom}$), 7.23 – 7.12 (m, 4H, $2H_{A\text{-}arom}$, $2H_{B\text{-}arom}$), 7.00 – 6.77 (m, 3H, $3H_{A\text{-}arom}$).**^{13}C NMR** (75 MHz, acetone-d_6) **δ** 172.41 (C_1), 152.31 ($C_{5'}$), 143.83 ($C_{q\text{-}indole}$), 135.95 ($C_{q\text{-}Barom}$), 134.47 ($CH_{B\text{-}arom}$), 132.35 (CH_{indole}), 132.18 ($C_{q\text{-}indole}$), 130.74 ($CH_{A\text{-}arom}$), 128.55 ($C_{q\text{-}B3}$), 127.84 (CH_{indole}), 127.24 ($CH_{B\text{-}arom}$), 126.08 (CH_{indole}), 125.15 (CH_{indole}), 122.61 ($CH_{A\text{-}arom}$), 115.60 ($CH_{A\text{-}arom}$), 114.72 ($C_{A\text{-}1}$), 113.14 ($CH_{B\text{-}arom}$), 97.08 (C_2). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3280 (NH), 1737 (C=O), 1659, 1619, 1600 (C=N), 1568, 1493, 1470, 1434, 1293, 1245, 1198, 1092, 923, 883. **ESI-MS m/z**: 376.2 $[M+H]^+$.



Synthesis of 7-chloro-3'-(4-chlorophenyl)-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one ***(26l)***

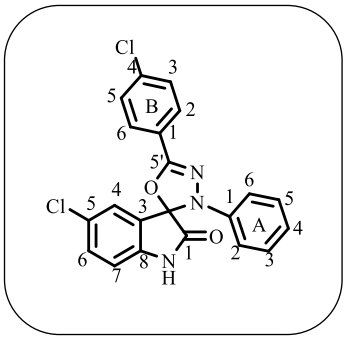
To a round bottom flask, was added 370.7 mg of 30h (1.4 mmol, 1.9 equiv) which was dissolved in 6 mL of dry CH_2Cl_2. Then, 135.6 mg of 7-chloroisatin (0.7 mmol, 1.0 equiv) was added and after 15 min of stirring, 168.0 µL of TEA (1.2 mmol, 2.0 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 10 mL of cold water and the organic layer was washed with 30 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The yellow oil was purified by flash chromatography, using as eluent gradient *n*-hexane to *n*-hexane: ethyl acetate (3:2) to afford, after recrystallization, a yellow solid (209.8 mg, 68%). **Mp:** 123-125ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.14 (br s, 1H, NH), 7.88 (m, 2H, H-A_{arom}), 7.77 (d, *J* = 2.1 Hz, 1H, H_{indole}), 7.67 (dd, *J* = 8.3, 2.1 Hz, 1H, H_{indole}), 7.56 (m, 2H, H-A_{arom}), 7.13 (m, 3H, H_{indole}, H-B_{arom}), 6.78 (m, 3H, H-B_{arom}). **^{13}C NMR** (75 MHz,



acetone-d_6) **δ** (ppm) 172.10 (C_1), 152.72 ($C_{5'}$), 143.59 ($C_{q\text{-indole}}$), 137.81 ($C_{q\text{-Aarom}}$), 137.23 (CH_{indole}), 130.85 ($CH_{B\text{-arom}}$), 130.76 (CH_{indole}), 130.71 ($CH_{A\text{-arom}}$), 129.38 ($CH_{A\text{-arom}}$), 127.49 ($C_{q\text{-indole}}$), 125.26 ($C_{q\text{-A4}}$), 122.78 ($CH_{B\text{-arom}}$), 116.99 ($C_{q\text{-7}}$), 115.60 ($CH_{B\text{-arom}}$), 115.13 (CH_{indole}), 114.71 ($C_{B\text{-1}}$), 96.57 (C_2). **IR** (NaCl) $\boldsymbol{\nu_{max}}$ (cm^{-1}) 3273 (NH), 1745 (C=O), 1651, 1599 (C=N), 1489, 1406, 1373, 1290, 1251, 1190, 1103, 1091, 1016, 1014, 906, 846, 831. **ESI-MS m/z**: 410.2 $[M+H]^+$.

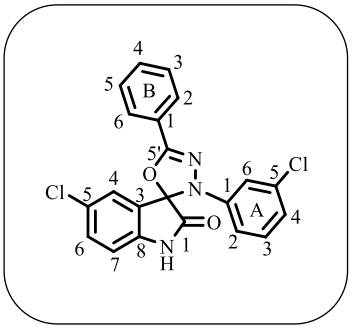
*Synthesis of 5-chloro-5'-(4-chlorophenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26m**)*

To a round bottom flask, was added 312.5 mg of 30b (1.2 mmol, 1.9 equiv) which was dissolved in 6 mL of dry CH_2Cl_2. Then, 115.8 mg of 5-chloroisatin (0.6 mmol, 1.0 equiv) was added and after 15 min of stirring, 168.0 µL of TEA (1.2 mmol, 1.9 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 10 mL of cold water and the organic layer was washed with 30 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The yellow oil was purified by flash chromatography, using as eluent gradient n-hexane to n-hexane: ethyl acetate (3:2) to afford, after recrystallization, a yellow solid (167.2 mg, 68%). **Mp:** 130-131ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.14 (br s, 1H, NH), 7.89 (d, *J* = 8.7 Hz, 2H, H-B_{arom}), 7.65 (d, *J* = 2.1 Hz, 1H, H_{indole}), 7.56 (m, 2H, H-B_{arom}), 7.53 (d, *J* = 2.2 Hz, 1H, H_{indole}), 7.17 (m, 3H, H_{indole}, H-A_{arom}), 6.78 (m, 3H, H-A_{arom}). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm)172.26 (C_1), 152.72 ($C_{5'}$), 143.74 ($C_{q\text{-indole}}$), 143.15 ($C_{q\text{-indole}}$), 137.82 ($C_{q\text{-B}}$), 134.33 (CH_{indole}), 130.85 ($CH_{A\text{-arom}}$), 130.72 ($CH_{B\text{-arom}}$), 129.87 ($C_{q\text{-indole}}$), 129.39 ($CH_{B\text{-arom}}$), 127.98 (CH_{indole}), 127.14 ($C_{A\text{-1}}$), 125.26 ($C_{q\text{-B4}}$), 122.78 ($CH_{A\text{-arom}}$), 115.62 ($CH_{A\text{-arom}}$), 114.70 (CH_{indole}), 96.65 (C_2). **IR** (NaCl) $\boldsymbol{\nu_{max}}$ (cm^{-1}) 3275 (NH), 1743 (C=O), 1599 (C=N), 1489, 1406, 1373, 1253, 1249, 1199, 1093, 1091, 1012, 839, 831. **ESI-MS m/z**: 410.2 $[M+H]^+$.



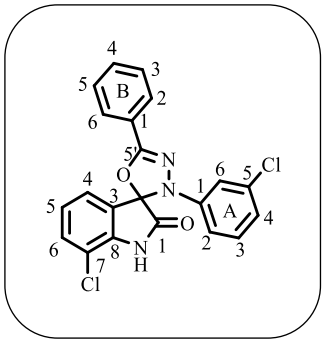
*Synthesis of 5-chloro-3'-(3-chlorophenyl)-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26n**)*

To a round bottom flask, was added 361.8 mg of 30a (1.4 mmol, 2.2 equiv) which was dissolved in 6 mL of dry CH2CL2. Then, 111.3 mg of 5-chloroisatin (0.6 mmol, 1.0 equiv) was added and after 15 min of stirring, 168.0 µL of TEA (1.2 mmol, 2.0 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 10 mL of cold water and the organic layer was washed with 30 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The yellow oil was purified by flash chromatography, using as eluent gradient *n*-hexane to *n*-hexane: ethyl acetate (4:1) to afford, after recrystallization, a yellow solid (160.3 mg, 64%). **Mp:** 209-210ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.18 (br s, 1H, NH), 7.91 (dd, *J* = 7.7, 1.9 Hz, 2H, H-B_{arom}), 7.68 (d, *J* = 2.1 Hz, 1H, H_{indole}), 7.51 (m, 4H, H-B_{arom}, H_{indole}), 7.15 (m, 2H, H_{indole}, H-A_{arom}), 7.09 (t, *J* = 2.1 Hz, 1H, H-A_{arom}), 6.86 (ddd, *J* = 8.2, 1.9, 0.7 Hz, 1H, H-A_2), 6.64 (ddd, *J* = 8.3, 2.2, 0.6 Hz, 1H, H-A_{arom}). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm) 171.96 (C_1), 155.23 ($C_{5'}$), 145.06 ($C_{q\text{-}A}$), 143.15 ($C_{q\text{-}A}$), 136.28 ($CH_{B\text{-}1}$), 134.57 (CH_{indole}), 132.36 (CH_{indole}), 130.54 ($CH_{B\text{-}arom}$), 130.10 ($C_{q\text{-}indole}$), 128.04 ($CH_{B\text{-}arom}$), 126.17 ($C_{q\text{-}indole}$), 126.17 ($C_{q\text{-}indole}$), 122.21 ($CH_{A\text{-}arom}$), 115.61 ($CH_{A\text{-}arom}$), 114.81 ($CH_{A\text{-}arom}$), 113.30 ($CH_{A\text{-}arom}$), 96.28 (C_2). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3271 (NH), 1743 (C=O), 1593 (C=N), 1481, 1448, 1376, 1313, 1265, 1201, 1141, 1097, 1068, 1024, 821. **ESI-MS m/z**: 410.2 $[M+H]^+$.



*Synthesis of 7-chloro-3'-(3-chlorophenyl)-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26o**)*

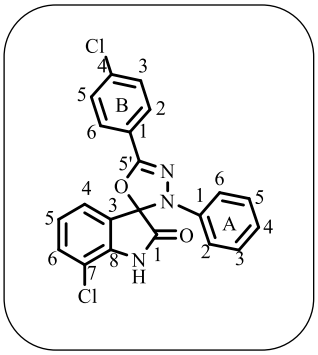
To a round bottom flask, was added 96.9 mg of 30a (0.4 mmol, 2.3 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 29.4 mg of 7-chloroisatin (0.2 mmol, 1.0 equiv) was added and after 15 min of stirring, 44.0 µL of TEA (0.3 mmol, 2.0 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The yellow oil was purified by preparative TLC, using as eluent *n*-hexane: ethyl acetate (8:2) to afford, after recrystallization, a yellow solid (45.4 mg, 68%). **Mp:** 205-207ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.43 (br s, 1H, NH), 7.91 (dd, *J* = 7.8, 1.8 Hz, 2H, H-B_{arom}), 7.55 (m, 5H, H_{indole}, H-B_{arom}), 7.18 (dd, *J* = 7.9, 3.0 Hz, 1H, H_{indole}), 7.15 (br s, 1H, H-A_{arom}), 7.10 (t, *J* =



2.1 Hz, 1H, H-A_{arom}), 6.87 (dd, *J* = 7.9, 1.9 Hz, 1H, H-A_{arom}), 6.61 (dd, *J* = 8.3, 1.5 Hz, 1H, H-A_{arom}). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm) 172.04 (C_1), 154.29 ($C_{5'}$), 145.10 ($C_{q\text{-}Aarom}$), 136.30 ($C_{q\text{-}indole}$), 134.51 (CH_{indole}), 132.90 ($CH_{B\text{-}arom}$), 132.40 (CH_{indole}), 130.58 ($CH_{B\text{-}arom}$), 128.04 ($CH_{B\text{-}arom}$), 126.69 ($C_{q\text{-}Aarom}$), 126.55 ($CH_{A\text{-}arom}$), 126.45 ($CH_{A\text{-}arom}$), 126.11 ($C_{q\text{-}indole}$), 122.30 ($CH_{A\text{-}arom}$), 118.00 ($C_{q\text{-}indole}$), 115.77 ($CH_{A\text{-}arom}$), 113.36 ($CH_{A\text{-}arom}$), 96.83 (C_2). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3381 (NH), 1747 (C=O), 1622, 1595 (C=N), 1593, 1475, 1450, 1373, 1302, 1263, 1186, 1145, 1066, 1060, 1024. **ESI-MS m/z**: 410.2 $[M+H]^+$.

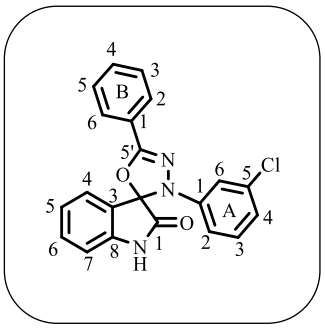
*Synthesis of 7-chloro-5'-(4-chlorophenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26p**)*

To a round bottom flask, was added 168.3 mg of 30b (0.6 mmol, 2.0 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 57.0 mg of 7-chloroisatin (0.3 mmol, 1.0 equiv) was added and after 15 min of stirring, 86.0 µL of TEA (0.6 mmol, 2.0 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The yellow oil was purified by flash chromatography, using as eluent gradient *n*-hexane to *n*-hexane: ethyl acetate (7:3) to afford, after recrystallization, a yellow solid (85.8 mg, 67%). **Mp:** 229-231ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.44 (br s, 1H, NH), 7.88 (d, *J* = 8.5 Hz, 2H, $2H_{B\text{-}arom}$) 7.51 (m, 4H, $2H_{Aarom}$, $2H_{indole}$), 7.16 (m, 3H, $2H_{B\text{-}arom}$, H_{indole}), 6.84 (m, 3H, $3H_{A\text{-}arom}$). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm) 172.34 (C_1), 152.76 ($C_{5'}$), 143.75 ($C_{q\text{-}indole}$), 141.99($C_{q\text{-}indole}$), 137.87 ($C_{q\text{-}indole}$), 134.28 (CH_{indole}), 130.86 (CH_{indole}), 130.75 ($CH_{A\text{-}arom}$), 129.37 ($CH_{B\text{-}arom}$), 127.09 ($C_{q\text{-}B1}$), 126.36 ($CH_{B\text{-}arom}$), 125.18 ($C_{q\text{-}A1}$), 122.85 ($CH_{A\text{-}arom}$), 117.83 ($C_{q\text{-}B4}$), 115.72 ($CH_{A\text{-}arom}$), 97.18 (C_2). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3227 (NH), 1747 (C=O), 1620, 1599 (C=N), 1489, 1149, 1143, 1089, 1055, 1012, 833. **ESI-MS m/z**: 410.2 $[M+H]^+$.



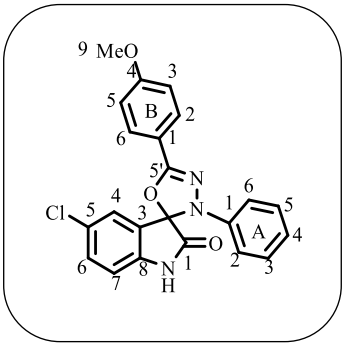
*Synthesis of 3'-(3-chlorophenyl)-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**2q**)*

To a round bottom flask, was added 308.0 mg of 30a (1.2 mmol, 3.3 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 51.9 mg of isatin (0.4 mmol, 1.0 equiv) was added and after 19 min of stirring, 150.0 µL of TEA (1.1 mmol, 3.1 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated to obtain, after recrystallization, a yellow solid which was purified by flash chromatography, using as eluent gradient *n*-hexane to *n*-hexane: ethyl acetate (8:2) to afford, after recrystallization, a yellow solid (115.4 mg, 87%). **Mp:** 216-217ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 10.08 (br s, 1H, NH), 7.90 (d, *J* = 5.0 Hz, 2H, H-B_{arom}), 7.51 (m, 5H, 2H_{indole}, 3H-B_{arom}), 7.12 (m, 3H, 2H_{indole}, H-A_{arom}), 7.05 (br s, 1H, H-A_{arom}), 6.83 (d, *J* = 7.7 Hz, 1H, H-A_{arom}), 6.63 (d, *J* = 7.8 Hz, 1H, H-A_{arom}). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm) 171.44 (C_1), 155.39 ($C_{5'}$), 144.49 ($C_{q\text{-indole}}$), 143.62 ($C_{q\text{-indole}}$), 135.90 ($C_{q\text{-}1}$), 133.94 ($C_{B\text{-}1}$), 132.402 (CH_{Barom}), 131.52 (CH_{indole}), 129.80 ($CH_{B\text{-arom}}$), 127.20 ($CH_{B\text{-arom}}$), 127.13 (CH_{indole}), 125.53 ($C_{A\text{-}5}$), 124.59 (CH_{indole}), 121.14 ($CH_{A\text{-arom}}$), 114.64 ($CH_{A\text{-arom}}$), 112.47 ($CH_{A\text{-arom}}$), 97.82 (C_2). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3257 (NH), 1741 (C=O), 1622, 1593 (C=N), 1483, 1450, 1273, 1201, 1089,1021, 945, 860. **ESI-MS m/z**: 376.2 $[M+H]^+$.



*Synthesis of 5-chloro-5'-(4-methoxyphenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26r**)*

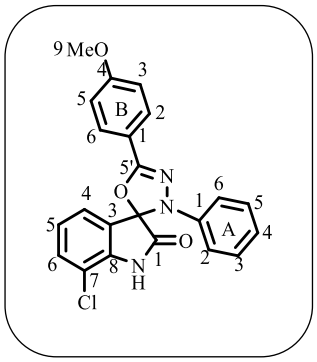
To a round bottom flask, was added 161.9 mg of 30d (0.6 mmol, 1.9 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 59.1 mg of 5-chloroisatin (0.3 mmol, 1.0 equiv) was added and after 15 min of stirring, 84.0 µL of TEA (0.6 mmol, 1.9 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The brown oil was purified by flash chromatography, using as eluent gradient *n*-hexane to *n*-hexane: ethyl acetate (7:3) to afford, after recrystallization, a yellow solid (82.2 mg, 63%). **Mp:** 156-157ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 7.99 (d, *J* = 8.8 Hz, 2H, 2$H_{B\text{-arom}}$), 7.66 – 7.60 (m, 1H, H_{indole}), 7.55 (s, 1H, H_{indole}), 7.22 – 7.12 (m, 2H, 2$H_{B\text{-arom}}$), 7.07 (dd, *J* = 13.8, 5.0 Hz, 3H, $H_{A\text{-arom}}$, H_{indole}), 6.95 (d, *J* = 7.7 Hz, 2H, 2$H_{A\text{-arom}}$), 6.79



(t, *J* = 7.3 Hz, 1H, $H_{A\text{-}arom}$), 3.89 (s, 3H, H_9). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** 184.69 (C_2), 167.48 ($C_{5'}$), 164.17 ($C_{q\text{-}indole}$), 151.63 ($C_{q\text{-}Barom}$), 150.91 ($C_{q\text{-}Barom}$), 139.11 (CH_{indole}), 130.76 ($CH_{B\text{-}arom}$), 130.36 ($CH_{B\text{-}arom}$), 129.46 ($C_{q\text{-}indole}$), 127.04 ($C_{q\text{-}indole}$), 125.79 (CH_{indole}), 121.16 ($CH_{A\text{-}arom}$), 120.90 ($C_{A\text{-}1}$), 115.57 (CH_{indole}), 115.28 ($CH_{A\text{-}arom}$), 114.71 ($CH_{A\text{-}arom}$), 96.65 (C_2), 56.57 ($CH_{3\text{-}B9}$). **IR** (NaCl) **ν_{max}** (cm^{-1}) 2419 (NH), 1743 (C=O), 1608, 1604 (C=N), 1494, 1303, 1251, 1174, 1026, 896, 842.

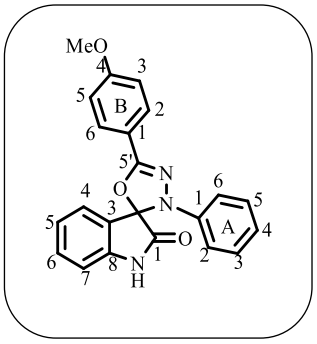
*Synthesis of 7-chloro-5'-(4-methoxyphenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26s**)*

To a round bottom flask, was added 171.1 mg of 30d (0.7 mmol, 2.0 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 59.7 mg of 7-chloroisatin (0.3 mmol, 1.0 equiv) was added and after 15 min of stirring, 86.0 µL of TEA (0.6 mmol, 1.9 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The obtained oil was purified by flash chromatography, using as eluent gradient *n*-hexane to *n*-hexane: ethyl acetate (7:3) to afford, after recrystallization, a yellow solid (48.6 mg, 53%). **Mp:** 213-215ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** 10.28 (s, 1H, NH), 7.86 – 7.80 (m, 2H, $H_{B\text{-}arom}$), 7.69 (dd, *J* = 8.1, 1.1 Hz, 1H, H_{indole}), 7.55 (ddd, *J* = 7.5, 3.1, 1.1 Hz, 1H, H_{indole}), 7.49 (dd, *J* = 7.5, 1.1 Hz, 1H, H_{indole}), 7.21 – 7.14 (m, 2H, $H_{B\text{-}arom}$), 7.11 – 7.05 (m, 2H, $H_{A\text{-}arom}$), 6.91 – 6.81 (m, 2H, $H_{A\text{-}arom}$, $H_{A\text{-}arom}$), 3.90 (s, 3H, $3H_{B9}$). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** 171.76 (C_1), 163.68 ($C_{q\text{-}Barom}$), 153.68 ($C_{5'}$), 144.32 ($C_{q\text{-}indole}$), 139.25 (CH_{indole}), 134.09 (CH_{indole}), 130.78 ($CH_{B\text{-}arom}$), 129.65 ($CH_{B\text{-}arom}$), 127.54 ($C_{q\text{-}Barom}$), 126.28 (CH_{indole}), 125.68 ($C_{q\text{-}indole}$), 124.72 ($C_{q\text{-}indole}$), 122.48 ($CH_{A\text{-}arom}$), 118.76 ($C_{q\text{-}Aarom}$), 117.79 ($C_{q\text{-}7}$)115.98 ($CH_{A\text{-}arom}$), 115.74 ($CH_{A\text{-}arom}$), 96.74 (C_2), 56.67 ($CH_{3\text{-}B9}$). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3419 (NH), 1743 (C=O), 1643, 1604 (C=N), 1494, 1265, 1251, 1174, 1026, 900, 846. **ESI-MS m/z**: 406.2 $[M+H]^+$.



*Synthesis of 5'-(4-methoxyphenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26t**)*

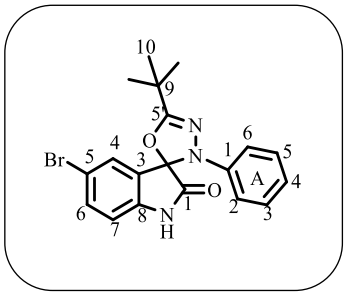
To a round bottom flask, was added 192.6 mg of 30d (0.7 mmol, 1.9 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 57.1 mg of isatin (0.4 mmol, 1.0 equiv) was added and after 15 min of stirring, 86.0 µL of TEA (0.6 mmol, 1.9 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried



with anhydrous sodium sulphate and the solvent was evaporated. The obtained oil was purified by flash chromatography, using as eluent gradient *n*-hexane to *n*-hexane: ethyl acetate (7:3) to afford, after recrystallization, a yellow solid (38.2 mg, 42%). **Mp:** 224-226ºC **^{1}H NMR** (300 MHz, acetone-d_6) **δ** 9.89 (s, 1H, NH), 7.87 – 7.81 (m, 2H, $2H_{B\text{-}arom}$), 7.53 – 7.46 (m, 2H, $2H_{indole}$), 7.19 – 7.02 (m, 6H, $2H_{A\text{-}arom}$, $2H_{B\text{-}arom}$, $2H_{indole}$), 6.91 – 6.77 (m, 3H, $3H_{A\text{-}arom}$), 3.89 (s, 3H, $H_{B\text{-}7}$). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** 172.81 (C_1), 163.57 ($C_{5'}$), 153.64 ($C_{q\text{-}Barom}$), 144.42 ($C_{q\text{-}indole}$), 134.22 (CH_{indole}), 130.64 ($CH_{B\text{-}arom}$), 129.57 ($CH_{B\text{-}arom}$), 127.72 (CH_{indole}), 125.73 ($C_{q\text{-}Barom}$), 125.05 (CH_{indole}), 122.09 ($CH_{A\text{-}arom}$), 119.03 ($C_{q\text{-}indole}$), 115.94 ($CH_{A\text{-}arom}$), 115.55 ($CH_{A\text{-}arom}$), 115.33 ($C_{A\text{-}1}$), 113.04 (CH_{indole}), 96.65 (C_2), 56.65 ($CH_{3\text{-}B7}$). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3419 (NH), 1743 (C=O), 1606 (C=N), 1498, 1257, 1197, 1142, 1122, 1084, 1022, 837. **ESI-MS m/z**: 372.3 $[M+H]^+$.

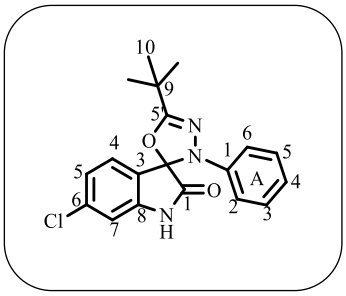
*Synthesis of 5-bromo-5'-(tert-butyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26u**)*

To a round bottom flask, was added 140.4 mg of 30i (0.7 mmol, 2.2 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 67.1 mg of 5-bromoisatin (0.3 mmol, 1.0 equiv) was added and after 15 min of stirring, 84.0 µL of TEA (0.6 mmol, 2.0 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The obtained oil was purified by flash chromatography, using as eluent *n*-hexane: ethyl acetate (7:3) to afford, after recrystallization, a yellow solid (107.2 mg, 90%). **Mp:** 210-212ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 9.83 (s, 1H, NH), 7.67 – 7.50 (m, 2H, H_{indole}), 7.45 – 7.23 (m, 2H, H_{indole}, $H_{A\text{-}arom}$), 7.19 – 6.98 (m, 2H, $2H_{A\text{-}arom}$), 6.91 – 6.69 (m, 2H, $2H_{A\text{-}arom}$), 1.35 – 1.21 (m, 9H, $9H_{10}$). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** 170.44 (C_1), 154.35 ($C_{5'}$), 144.75 ($C_{q\text{-}indole}$), 136.82 (CH_{indole}), 136.20 (CH_{indole}), 130.32 ($CH_{A\text{-}arom}$), 127.30 (CH_{indole}), 121.91 ($C_{q\text{-}indole}$), 121.56 ($CH_{A\text{-}arom}$), 116.78 ($C_{q\text{-}indole}$), 116.17 ($C_{q\text{-}Aarom}$), 115.52 ($CH_{A\text{-}arom}$), 114.93 ($CH_{A\text{-}arom}$), 96.19 (C_2), 39.66 (C_9), 28.35 ($CH_{3\text{-}10}$). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3275 (NH), 2970 (alkyl CH), 1743 (C=O), 1658, 1599 (C=N), 1562, 1500, 1452, 1288, 1203, 1145, 1093, 937, 875, 825.



*Synthesis of 5'-(tert-butyl)-6-chloro-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26v**)*

To a round bottom flask, was added 143.8 mg of 30i (0.7 mmol, 2.2 equiv) which was dissolved in 3 mL of dry CH_2Cl_2. Then, 55.3 mg of 6-chloroisatin (0.3 mmol, 1.0 equiv) was added and after 15 min of stirring, 84.0 µL of TEA (0.6 mmol, 2.0 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 5 mL of cold water and the organic layer was washed with 15 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The obtained oil was purified by flash chromatography, using as eluent gradient *n*-hexane to *n*-hexane: ethyl acetate (4:1). to afford, after recrystallization, a yellow solid (93.0 mg, 86%). **Mp:** 222-223ºC. **^{1}H NMR** (300 MHz, acetone-d_6) **δ** (ppm) 9.85 (d, *J* = 12.7 Hz, 1H, NH), 7.56 (m, 1H, H_7), 7.06 (m, 4H, $H_{A\text{-}arom}$), 6.73 (m, 3H, $2H_{indole}$, $H_{A\text{-}arom}$), 1.23 (m, 9H, $9H_{10}$). **^{13}C NMR** (75 MHz, acetone-d_6) **δ** (ppm) 171.71 (C_1), 155.95($C_{5'}$), 138.33 ($C_{q\text{-}indole}$), 130.40 ($C_{qA\text{-}arom}$), 130.10 ($CH_{A\text{-}arom}$), 130.02 ($CH_{A\text{-}arom}$), 128.98, 125.58 (CH_{indole}), 124.47 (CH_{indole}), 121.84 ($C_{q\text{-}indole}$), 121.51($C_{q\text{-}indole}$), 119.99 (CH_{indole}), 116.25 ($CH_{A\text{-}arom}$), 93.14 (C_2), 39.65 (C_9)28.26 (C_{10}). **IR** (NaCl) **ν_{max}** (cm^{-1}) 3219 (NH), 2970 (alkyl CH), 1747 (C=O), 1662, 1599 (C=N), 1556, 1502, 1446, 1369, 1288, 1199, 1145, 1091, 1072, 1019, 914, 879, 812.



*Synthesis of 5-bromo-3',5'-diphenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (**26w**)*

To a round bottom flask, was added 51.0 mg of 30g (0.2 mmol, 2.0 equiv) which was dissolved in 6 mL of dry CH_2CL_2. Then, 25.0 mg of 5-bromoisatin (0.1 mmol, 1.0 equiv) was added and after 15 min of stirring, 15.4 µL of TEA (0.2 mmol, 2.0 equiv) was added dropwise. The reaction was stirred overnight under N_2 atmosphere. The reaction was quenched with 10 mL of cold water and the organic layer was washed with 30 mL of brine twice. The resulting solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The obtained oil was purified by flash chromatography using a *n*-hexane: ethyl acetate (3:2) as eluent to obtain, after recrystallization, a yellow solid (31.1mg, 67%). The NMR is in accordance with the one described in the literature.[18]

6.1.5. General procedure for the microwave-assisted synthesis of spirooxadiazoline oxindoles **26**

In a microwave vessel, the 5-bromoisatin (2.0 equiv) was added with the hydrazones **30a-g** (1.0 equiv) and the vessel was introduced to the microwave. The reactions were tested from 90ºC

to 180ºC, the power and the pressure were set at maximum 300 W and 300 bar, respectively. After cooling, the reaction was quenched with cold water and the organic layer was extracted with brine twice. The organic layer was concentrated and purified by flash chromatography.

6.2. Biology

6.2.1. Anti-proliferative assays

In breast cancer cell lines, cytotoxicity was assessed using 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H-tetrazolium bromide (MTT), a yellow, water soluble tetrazolium dye that is converted by mitochondrial dehydrogenases in viable cells to a water-insoluble, purple formazan.[89, 94] The day before experiments cells obtained from the American Type Culture Collection HEK 293T – human embryonic kidney epithelial cell line (ATCC HBT-22TM) were seeded at 2x104 cells per well in 96 well tissue culture plates, in 100 µl of RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units of penicillin G (sodium salt), 100 µg of streptomycin sulfate and 2 mM of L-glutamine, at a concentration that allows cells to grow exponentially during the assay.

Tested compounds were dissolved in dimethyl sulfoxide (DMSO) serially diluted in the culture medium and added to the cells. The final concentration of DMSO in culture medium during treatment did not exceed 0.5% (v/v), and the same concentration of DMSO was added to the control. Each compound concentration was tested in triplicate in a single experiment which was repeated at least 3 times, controls contained equivalent concentrations of DMSO. Doxorubicin was used as positive control at concentration of 4 µM. Cells were incubated at 37ºC in humidified 5% CO_2 atmosphere. After 48 h, cell media was removed and replaced with fresh medium containing the MTT dye at 0.5mg/mL and after 3 h of incubation, the media was removed and intracellular formazan crystals were solubilized and extracted with 100 µL of DMSO. After 15 minutes, at room temperature, absorbance was measured at 570 nm in a microplate reader (FLUOstar Omega, BMG Labtech, Germany) and the percentage of viable cells was determined for each compound concentration as described previously.[14] IC_{50}s were determined by non-linear regression using GraphPad PRISM software. Statistical analysis of the experimental data was performed by applying one-way ANOVA tests using the software package GraphPad PRISM. Differences were considered to be significant when $P < 0.05$.

6.2.2. *P. falciparum* assays

The screening against *Plasmodium falciparum* CQ-resistant and sensitive strains were performed by Dr. Philip Rosenthal group, according to the procedure previously reported.[95]

Chapter 7

REFERENCES

Chapter 7. References

[1] A.G. Atanasov, B. Waltenberger, E.-M. Pferschy-Wenzig, T. Linder, C. Wawrosch, P. Uhrin, V. Temml, L. Wang, S. Schwaiger, E.H. Heiss, J.M. Rollinger, D. Schuster, J.M. Breuss, V. Bochkov, M.D. Mihovilovic, B. Kopp, R. Bauer, V.M. Dirsch, H. Stuppner, Discovery and resupply of pharmacologically active plant-derived natural products: A review, Biotechnology Advances, 33 (2015) 1582-1614.

[2] M.-Z. Zhang, Q. Chen, G.-F. Yang, A review on recent developments of indole-containing antiviral agents, European Journal of Medicinal Chemistry, 89 (2015) 421-441.

[3] L. Katz, R.H. Baltz, Natural product discovery: past, present, and future, Journal of Industrial Microbiology & Biotechnology, 43 (2016) 155-176.

[4] G.M. Cragg, P.G. Grothaus, D.J. Newman, Impact of Natural Products on Developing New Anti-Cancer Agents, Chemical Reviews, 109 (2009) 3012-3043.

[5] G.M. Cragg, D.J. Newman, Natural products: A continuing source of novel drug leads, Biochimica et Biophysica Acta 1830 (2013) 3670-3695.

[6] W. Gul, M.T. Hamann, Indole alkaloid marine natural products: An established source of cancer drug leads with considerable promise for the control of parasitic, neurological and other diseases, Life Sciences, 78 (2005) 442-453.

[7] S.T. Hilton, T.C.T. Ho, G. Pljevaljcic, K. Jones, A New Route to Spirooxindoles, Organic Letters, 2 (2000) 2639-2641.

[8] M.T. El-sayed, N.A. Hamdy, D.A. Osman, K.M. Ahmed, Indoles as anticancer agents, Advances in Modern Oncology Research, 1 (2015) 20-35.

[9] M.T. El-sayed, N.A. Hamdy, D.A. Osman, K.M. Ahmed, Indoles as anticancer agents, 2015, 1 (2015) 16.

[10] M. Frederich, M. Tits, L. Angenot, Potential antimalarial activity of indole alkaloids, Transactions of the Royal Society of Tropical Medicine and Hygiene, 102 (2008) 11-19.

[11] C.V. Galliford, K.A. Scheidt, Pyrrolidinyl-Spirooxindole Natural Products as Inspirations for the Development of Potential Therapeutic Agents, Angewandte Chemie International Edition, 46 (2007) 8748-8758.

[12] C.J.A. Ribeiro, S. Praveen Kumar, R. Moreira, M.M.M. Santos, Efficient synthesis of spiroisoxazoline oxindoles, Tetrahedron Letters, 53 (2012) 281-284.

[13] C.J.A. Ribeiro, J.D. Amaral, C.M.P. Rodrigues, R. Moreira, M.M.M. Santos, Synthesis and evaluation of spiroisoxazoline oxindoles as anticancer agents, Bioorganic & Medicinal Chemistry, 22 (2014) 577-584.

[14] Â. Monteiro, L.M. Gonçalves, M.M.M. Santos, Synthesis of novel spiropyrazoline oxindoles and evaluation of cytotoxicity in cancer cell lines, European Journal of Medicinal Chemistry, 79 (2014) 266-272.

[15] R.C. Nunes, C.J.A. Ribeiro, Â. Monteiro, C.M.P. Rodrigues, J.D. Amaral, M.M.M. Santos, In vitro targeting of colon cancer cells using spiropyrazoline oxindoles, European Journal of Medicinal Chemistry, 139 (2017) 168-179.

[16] C.J.A. Ribeiro, J.D. Amaral, C.M.P. Rodrigues, R. Moreira, M.M.M. Santos, Spirooxadiazoline oxindoles with promising in vitro antitumor activities, MedChemComm, 7 (2016) 420-425.

[17] A. Alizadeh, L. Moafi, A Convenient 1, 3-Dipolar Cycloaddition Reaction for the Synthesis of Spirooxindoles and Some Other Spirocompounds Containing the 1, 3, 4-Oxadiazole Moiety, Helvetica Chimica Acta, 99 (2016) 457-461.

[18] D. Uyar, Spirooxadiazoline Oxindoles as Potential Anticancer Agents, in: Department of Tharmaceutical and Therapeutical Chemistry, University of Lisbon, Lisbon, 2016.

[19] M. Kaur, M. Singh, N. Chadha, O. Silakari, Oxindole: A chemical prism carrying plethora of therapeutic benefits, European Journal of Medicinal Chemistry, 123 (2016) 858-894.

[20] S. Oniga, A.E. Parvu, B. Tiperciuc, M. Palage, O. Oniga, The study of the anti-inflamatory activity of some thiazolyl-1,3,4-oxadiazolines and 5-carboxiethyl-2-hydrazon-4-methyl-thiazole derivatives Farmacia, 59 (2011) 44-50.

[21] S. Ke, F. Liu, N. Wang, Q. Yang, X. Qian, 1, 3, 4-Oxadiazoline derivatives as novel potential inhibitors targeting chitin biosynthesis: Design, synthesis and biological evaluation, Bioorganic & Medicinal Chemistry Letters, 19 (2009) 332-335.

[22] A.A. El-Emam, O.A. Al-Deeb, M. Al-Omar, J. Lehmann, Synthesis, antimicrobial, and anti-HIV-1 activity of certain 5-(1-adamantyl)-2-substituted thio-1, 3, 4-oxadiazoles and 5-(1-

adamantyl)-3-substituted aminomethyl-1, 3, 4-oxadiazoline-2-thiones, Bioorganic & Medicinal Chemistry, 12 (2004) 5107-5113.

[23] C.J.A. Ribeiro, Design and synthesis of small molecule modulators of p53, in: Department of Pharmaceutical and Therapeutical Chemistry, University of Lisbon, Lisbon, 2015.

[24] J. Ru Hwu, C. Chieh Lin, S. Hsien Chuang, K. Yung King, T.-R. Su, S.-C. Tsay, Aminyl and iminyl radicals from arylhydrazones in the photo-induced DNA cleavage, Bioorganic & Medicinal Chemistry, 12 (2004) 2509-2515.

[25] A. DeAngelis, D.-H. Wang, S.L. Buchwald, Mild and Rapid Pd-Catalyzed Cross-Coupling with Hydrazine in Continuous Flow: Application to the Synthesis of Functionalized Heterocycles, Angewandte Chemie International Edition, 52 (2013) 3434-3437.

[26] S.A.M. Metwally, T.A. Mohamed, O.S. Moustafa, Y.A. El-Ossaily, Reactions of 4-alkylidene (arylidene)-1-phenylpyrazolidine-3,5-dione, Chemistry of Heterocyclic Compounds, 43 (2007) 1131-1137.

[27] U.N. Yadav, G.S. Shankarling, Room temperature ionic liquid choline chloride–oxalic acid: A versatile catalyst for acid-catalyzed transformation in organic reactions, Journal of Molecular Liquids, 191 (2014) 137-141.

[28] B.l. Török, A. Sood, S. Bag, R. Tulsan, S. Ghosh, D. Borkin, A.R. Kennedy, M. Melanson, R. Madden, W. Zhou, Diaryl Hydrazones as Multifunctional Inhibitors of Amyloid Self-Assembly, Biochemistry, 52 (2013) 1137-1148.

[29] H.V. Patel, K.A. Vyas, S.P. Pandey, P.S. Fernandes, Facile synthesis of hydrazonyl halides by reaction of hydrazones with N-halosuccinimide-dimethyl sulfide complex, Tetrahedron, 52 (1996) 661-668.

[30] G. Wang, X. Liu, T. Huang, Y. Kuang, L. Lin, X. Feng, Asymmetric catalytic 1, 3-dipolar cycloaddition reaction of nitrile imines for the synthesis of chiral spiro-pyrazoline-oxindoles, Organic letters, 15 (2012) 76-79.

[31] S. Rossiter, A convenient synthesis of 3-methyleneoxindoles: cytotoxic metabolites of indole-3-acetic acids, Tetrahedron Letters, 43 (2002) 4671-4673.

[32] J. Azizian, K. Jadidi, M. Mehrdad, Y. Sarrafi, One Pot Synthesis of Some New Spiro [3 H-Indol-3, 5′(4′ H)-[1, 2, 4] Oxadiazol]-2-Ones and Bis [Spiro [3 H-Indol-3, 5′(4′ H)-[1, 2, 4] Oxadiazol]-2-Ones], Synthetic Communications, 30 (2000) 2309-2315.

[33] M. Pineiro, T.M. Pinho e Melo, Microwave-Assisted 1, 3-Dipolar Cycloaddition: an Eco-Friendly Approach to Five-Membered Heterocycles, European Journal of Organic Chemistry, 2009 (2009) 5287-5307.

[34] A. Díaz-Ortiz, A. de la Hoz, F. Langa, Microwave irradiation in solvent-free conditions: an eco-friendly methodology to prepare indazoles, pyrazolopyridines and bipyrazoles by cycloaddition reactions, Green Chemistry, 2 (2000) 165-172.

[35] A. Arrieta, J.R. Carrillo, F.P. Cossío, A. Díaz-Ortiz, M. JoséGómez-Escalonilla, A. de la Hoz, F. Langa, A. Moreno, Efficient tautomerization hydrazone-azomethine imine under microwave irradiation. Synthesis of [4, 3′] and [5, 3′] bipyrazoles, Tetrahedron, 54 (1998) 13167-13180.

[36] A.H. Shinde, S. Vidyacharan, D.S. Sharada, Microwave-assisted facile synthesis of [a]-annelated pyrazolopyrroloindoles via intramolecular azomethine imine 1,3-dipolar cycloaddition, Tetrahedron Letters, 55 (2014) 3064-3069.

[37] C.C. Harris, M. Hollstein, Clinical implications of the p53 tumor-suppressor gene, New England Journal of Medicine, 329 (1993) 1318-1327.

[38] J.-C. Carry, C. Garcia-Echeverria, Inhibitors of the p53/hdm2 protein–protein interaction—Path to the clinic, Bioorganic & Medicinal Chemistry Letters, 23 (2013) 2480-2485.

[39] S. Nag, X. Zhang, K. Srivenugopal, M.-H. Wang, W. Wang, R. Zhang, Targeting MDM2-p53 interaction for cancer therapy: are we there yet?, Current Medicinal Chemistry, 21 (2014) 553-574.

[40] S. Shangary, S. Wang, Targeting the MDM2-p53 interaction for cancer therapy, Clinical Cancer Research, 14 (2008) 5318-5324.

[41] K.H. Vousden, X. Lu, Live or let die: the cell's response to p53, Nature reviews. Cancer, 2 (2002) 594.

[42] X. Wu, J.H. Bayle, D. Olson, A.J. Levine, The p53-MDM2 autoregulatory feedback loop, Genes & Development, 7 (1993) 1126-1132.

[43] L.T. Vassilev, p53 Activation by small molecules: application in oncology, Journal of Medicinal Chemistry, 48 (2005) 4491-4499.

[44] Y. Zhao, D. Bernard, S. Wang, Small molecule inhibitors of MDM2-p53 and MDMX-p53 interactions as new cancer therapeutics, BioDiscovery, 8 (2013).

[45] S.W. Lowe, S. Bodis, A. McClatchey, L. Remington, H.E. Ruley, D.E. Fisher, D.E. Housman, T. Jacks, p53 status and the efficacy of cancer therapy in vivo, Science, (1994) 807-807.

[46] K.T. Bieging, S.S. Mello, L.D. Attardi, Unravelling mechanisms of p53-mediated tumour suppression, Nature Reviews. Cancer, 14 (2014) 359-370.

[47] G. Karan, H. Wang, A. Chakrabarti, S. Karan, Z. Liu, Z. Xia, M. Gundluru, S. Moreton, Y. Sauntharajah, M.W. Jackson, Identification of a small molecule that overcomes HDMX-mediated suppression of p53, Molecular Cancer Therapeutics, 15 (2016) 574-582.

[48] X. Yu, S. Narayanan, A. Vazquez, D.R. Carpizo, Small molecule compounds targeting the p53 pathway: are we finally making progress?, Apoptosis, 19 (2014) 1055-1068.

[49] Y. Barak, T. Juven, R. Haffner, M. Oren, mdm2 expression is induced by wild type p53 activity, The EMBO journal, 12 (1993) 461.

[50] C. Prives, P.A. Hall, The p53 pathway, The Journal of Pathology, 187 (1999) 112-126.

[51] A. Böttger, V. Böttger, A. Sparks, W.-L. Liu, S.F. Howard, D.P. Lane, Design of a synthetic Mdm2-binding mini protein that activates the p53 response in vivo, Current Biology, 7 (1997) 860-869.

[52] R. Honda, H. Tanaka, H. Yasuda, Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53, FEBS letters, 420 (1997) 25-27.

[53] J. Roth, M. Dobbelstein, D.A. Freedman, T. Shenk, A.J. Levine, Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein, The EMBO Journal, 17 (1998) 554-564.

[54] K.L.J. Bill, J. Garnett, I. Meaux, X. Ma, C.J. Creighton, S. Bolshakov, C. Barriere, L. Debussche, A.J. Lazar, B.C. Prudner, SAR405838: A novel and potent inhibitor of the MDM2: p53 axis for the treatment of dedifferentiated liposarcoma, Clinical Cancer Research, 22 (2016) 1150-1160.

[55] G.C. Tortora, R.; Damiano, V.; Bianco, R.; Chen, J.; Agrawal, S.; Bianco, A.R.; Ciardiello, F. , A novel MDM2 antisense oligonucleotide has anti-tumor activity and potentiates cytotoxic drugs acting by different mechanisms in human colon cancer, International Journal of Cancer, 88 (2000) 804-809.

[56] H.O. Wang, P.; Zhang, Z.; Agrawal, S.; Zhang, R., Chemosensitization and radiosensitization of human cancer by antisense anti-MDM2 oligonucleotides: in vitro and in vivo activities and mechanisms, Annals of the New York Academy of Sciences, 1002 (2003) 217-235.

[57] H.Z. Wang, X.; Oliver, P.; Le, L.P.; Chen, J.; Chen, L.; Zhou, W.; Agrawal, S.; Zhang, R., MDM2 oncogene as a target for cancer therapy: An antisense approach, International Journal of Oncology, 15 (1999) 653-660.

[58] Y. Haupt, R. Maya, A. Kazaz, M. Oren, MDM2 promotes the rapid degradation of p53, Nature, 387 (1997) 296.

[59] Y. Zhao, A. Aguilar, D. Bernard, S. Wang, Small-Molecule Inhibitors of the MDM2–p53 Protein–Protein Interaction (MDM2 Inhibitors) in Clinical Trials for Cancer Treatment, Journal of Medicinal Chemistry, 58 (2015) 1038-1052.

[60] C.J. Ribeiro, J.D. Amaral, C.M. Rodrigues, R. Moreira, M.M. Santos, Spirooxadiazoline oxindoles with promising in vitro antitumor activities, MedChemComm, 7 (2016) 420-425.

[61] H.W. Cleator S., Coombes RC, Triple-negative breast cancer: Therapy options, Lancet Oncology, 8 (2007) 235-244.

[62] C.L. Anders CK, Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer, Clinical Breast Cancer, 9 (2009) 73-81.

[63] K.B. Horwitz, M.E. Costlow, W.L. McGuire, MCF-7: A human breast cancer cell line with estrogen, androgen, progesterone, and glucocorticoid receptors, Steroids, 26 (1975) 785-795.

[64] Patricia A.J. Muller, Karen H. Vousden, Mutant p53 in Cancer: New Functions and Therapeutic Opportunities, Cancer Cell, 25 (2014) 304-317.

[65] S. Vangapandu, M. Jain, K. Kaur, P. Patil, S.R. Patel, R. Jain, Recent advances in antimalarial drug development, Medicinal Research Reviews, 27 (2007) 65-107.

[66] N.J. White, Antimalarial drug resistance, Journal of Clinical Investigation, 113 (2004) 1084.

[67] WHO, Malaria, in, http://www.who.int/mediacentre/factsheets/fs094/en/, 2017.

[68] K. Singh, J. Okombo, C. Brunschwig, F. Ndubi, L. Barnard, C. Wilkinson, P.M. Njogu, M. Njoroge, L. Laing, M. Machado, Antimalarial Pyrido [1, 2-a] benzimidazoles: Lead Optimization, Parasite Life Cycle Stage Profile, Mechanistic Evaluation, Killing Kinetics, and in Vivo Oral Efficacy in a Mouse Model, Journal of Medicinal Chemistry, 60 (2017) 1432-1448.

[69] G.V. Brown, H.P. Beck, M. Molyneux, K. Marsh, Molecular Approaches to Epidemiology and Clinical Aspects of Malaria, Parasitology Today, 16 (2000) 448-451.

[70] H. Turner, Spiroindolone NITD609 is a novel antimalarial drug that targets the P-type ATPase PfATP4, Future Medicinal Chemistry, 8 (2016) 227-238.

[71] M. Rottmann, C. McNamara, B.K.S. Yeung, M.C.S. Lee, B. Zou, B. Russell, P. Seitz, D.M. Plouffe, N.V. Dharia, J. Tan, S.B. Cohen, K.R. Spencer, G.E. González-Páez, S.B. Lakshminarayana, A. Goh, R. Suwanarusk, T. Jegla, E.K. Schmitt, H.-P. Beck, R. Brun, F. Nosten, L. Renia, V. Dartois, T.H. Keller, D.A. Fidock, E.A. Winzeler, T.T. Diagana, Spiroindolones, a Potent Compound Class for the Treatment of Malaria, Science, 329 (2010) 1175-1180.

[72] T. Mita, A. Kaneko, J.K. Lum, B. Bwijo, M. Takechi, I.L. Zungu, T. Tsukahara, K. Tanabe, T. Kobayakawa, A. Björkman, Recovery of chloroquine sensitivity and low prevalence of the Plasmodium falciparum chloroquine resistance transporter gene mutation K76T following the discontinuance of chloroquine use in Malawi, Am. J. Trop. Med. Hyg., 68 (2003) 413-415.

[73] M. Mishra, V.K. Mishra, V. Kashaw, A.K. Iyer, S.K. Kashaw, Comprehensive review on various strategies for antimalarial drug discovery, European Journal of Medicinal Chemistry, 125 (2017) 1300-1320.

[74] T. Mita, K. Tanabe, K. Kita, Spread and evolution of Plasmodium falciparum drug resistance, Parasitology International, 58 (2009) 201-209.

[75] R.G. Ridley, W. Hofheinz, H. Matile, C. Jaquet, A. Dorn, R. Masciadri, S. Jolidon, W.F. Richter, A. Guenzi, M.A. Girometta, H. Urwyler, W. Huber, S. Thaithong, W. Peters, 4-Aminoquinoline analogs of chloroquine with shortened side chains retain activity against chloroquine-resistant Plasmodium falciparum, Antimicrobial Agents and Chemotherapy, 40 (1996) 1846-1854.

[76] B. Meunier, Hybrid Molecules with a Dual Mode of Action: Dream or Reality?, Accounts of Chemical Research, 41 (2008) 69-77.

[77] F. Benoit-Vical, J. Lelièvre, A. Berry, C. Deymier, O. Dechy-Cabaret, J. Cazelles, C. Loup, A. Robert, J.-F. Magnaval, B. Meunier, Trioxaquines Are New Antimalarial Agents Active on All Erythrocytic Forms, Including Gametocytes, Antimicrobial Agents and Chemotherapy, 51 (2007) 1463-1472.

[78] S.A.L. Laurent, C. Loup, S. Mourgues, A. Robert, B. Meunier, Heme Alkylation by Artesunic Acid and Trioxaquine DU1301, Two Antimalarial Trioxanes, ChemBioChem, 6 (2005) 653-658.

[79] T.J. Egan, H.M. Marques, The role of haem in the activity of chloroquine and related antimalarial drugs, Coordination Chemistry Reviews, 190 (1999) 493-517.

[80] G.L. Nixon, D.M. Moss, A.E. Shone, D.G. Lalloo, N. Fisher, P.M. O'Neill, S.A. Ward, G.A. Biagini, Antimalarial pharmacology and therapeutics of atovaquone, Journal of Antimicrobial Chemotherapy, 68 (2013) 977-985.

[81] W. Hughes , G. Leoung , F. Kramer , S.A. Bozzette , S. Safrin , P. Frame , N. Clumeck , H. Masur , D. Lancaster , C. Chan , J. Lavelle , J. Rosenstock , J. Falloon , J. Feinberg , S. LaFon , M. Rogers , F. Sattler Comparison of Atovaquone (566C80) with Trimethoprim-Sulfamethoxazole to Treat Pneumocystis carinii Pneumonia in Patients with AIDS, New England Journal of Medicine, 328 (1993) 1521-1527.

[82] E. Fernández-Álvaro, W.D. Hong, G.L. Nixon, P.M. O'Neill, F. Calderón, Antimalarial Chemotherapy: Natural Product Inspired Development of Preclinical and Clinical Candidates with Diverse Mechanisms of Action, Journal of Medicinal Chemistry, 59 (2016) 5587-5603.

[83] A.-C. Uhlemann, S. Wittlin, H. Matile, L.Y. Bustamante, S. Krishna, Mechanism of Antimalarial Action of the Synthetic Trioxolane RBX11160 (OZ277), Antimicrobial Agents and Chemotherapy, 51 (2007) 667-672.

[84] X. Wang, Y. Dong, S. Wittlin, S.A. Charman, F.C.K. Chiu, J. Chollet, K. Katneni, J. Mannila, J. Morizzi, E. Ryan, C. Scheurer, J. Steuten, J. Santo Tomas, C. Snyder, J.L. Vennerstrom, Comparative Antimalarial Activities and ADME Profiles of Ozonides (1,2,4-trioxolanes) OZ277, OZ439, and Their 1,2-Dioxolane, 1,2,4-Trioxane, and 1,2,4,5-Tetraoxane Isosteres, Journal of Medicinal Chemistry, 56 (2013) 2547-2555.

[85] S.A. Charman, S. Arbe-Barnes, I.C. Bathurst, R. Brun, M. Campbell, W.N. Charman, F.C.K. Chiu, J. Chollet, J.C. Craft, D.J. Creek, Y. Dong, H. Matile, M. Maurer, J. Morizzi, T. Nguyen, P. Papastogiannidis, C. Scheurer, D.M. Shackleford, K. Sriraghavan, L. Stingelin, Y. Tang, H. Urwyler, X. Wang, K.L. White, S. Wittlin, L. Zhou, J.L. Vennerstrom, Synthetic ozonide drug candidate OZ439 offers new hope for a single-dose cure of uncomplicated malaria, Proceedings of the National Academy of Sciences, 108 (2011) 4400-4405.

[86] P. Olliaro, T.N.C. Wells, The Global Portfolio of New Antimalarial Medicines Under Development, Clinical Pharmacology & Therapeutics, 85 (2009) 584-595.

[87] B.K.S. Yeung, B. Zou, M. Rottmann, S.B. Lakshminarayana, S.H. Ang, S.Y. Leong, J. Tan, J. Wong, S. Keller-Maerki, C. Fischli, A. Goh, E.K. Schmitt, P. Krastel, E. Francotte, K. Kuhen, D. Plouffe, K. Henson, T. Wagner, E.A. Winzeler, F. Petersen, R. Brun, V. Dartois, T.T. Diagana, T.H. Keller, Spirotetrahydro β-Carbolines (Spiroindolones): A New Class of Potent and Orally Efficacious Compounds for the Treatment of Malaria, Journal of Medicinal Chemistry, 53 (2010) 5155-5164.

[88] P.W. Smith, T.T. Diagana, B.K.S. Yeung, Progressing the global antimalarial portfolio: finding drugs which target multiple Plasmodium life stages, Parasitology, 141 (2013) 66-76.

[89] A. Cadete, L. Figueiredo, R. Lopes, C. Calado, A. Almeida, L. Gonçalves, Development and characterization of a new plasmid delivery system based on chitosan–sodium deoxycholate nanoparticles, European Journal of Pharmaceutical Sciences, 45 (2012) 451-458.

[90] J.C. van Pelt-Koops, H.E. Pett, W. Graumans, M. van der Vegte-Bolmer, G.J. van Gemert, M. Rottmann, B.K.S. Yeung, T.T. Diagana, R.W. Sauerwein, The Spiroindolone Drug Candidate NITD609 Potently Inhibits Gametocytogenesis and Blocks Plasmodium falciparum Transmission to Anopheles Mosquito Vector, Antimicrobial Agents and Chemotherapy, 56 (2012) 3544-3548.

[91] B. Zou, P. Yap, L.-S. Sonntag, S.Y. Leong, B.K.S. Yeung, T.H. Keller, Mechanistic Study of the Spiroindolones: A New Class of Antimalarials, Molecules, 17 (2012) 10131.

[92] M.P. Crespo-Ortiz, M.Q. Wei, Antitumor activity of artemisinin and its derivatives: from a well-known antimalarial agent to a potential anticancer drug, BioMed Research International, 2012 (2011).

[93] A. Mahajan, S. Yeh, M. Nell, C.E.J. van Rensburg, K. Chibale, Synthesis of new 7-chloroquinolinyl thioureas and their biological investigation as potential antimalarial and anticancer agents, Bioorganic & Medicinal Chemistry Letters, 17 (2007) 5683-5685.

[94] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, Journal of Immunological Methods, 65 (1983) 55-63.

[95] S. Kumar, A. Saini, J. Gut, P.J. Rosenthal, R. Raj, V. Kumar, 4-Aminoquinoline-chalcone/-N-acetylpyrazoline conjugates: Synthesis and antiplasmodial evaluation, European Journal of Medicinal Chemistry, 138 (2017) 993-1001.